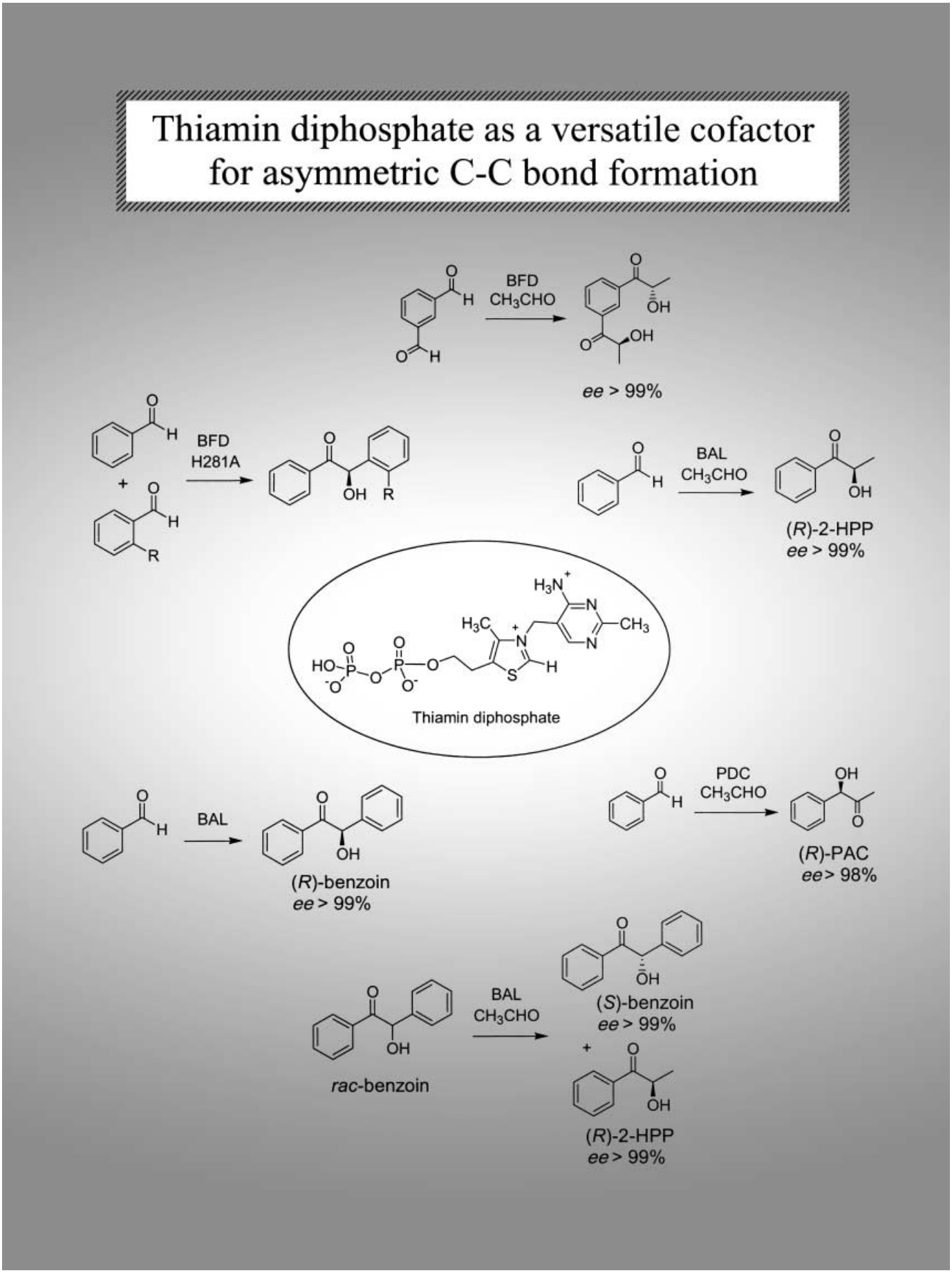

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Thiamin-Diphosphate-Dependent Enzymes: New Aspects of Asymmetric C–C Bond Formation

Martina Pohl,[b] Bettina Lingen,[b] and Michael Müller*[a]

Dedicated to Professor Maria-Regina Kula on the occasion of her 65th birthday.

Abstract: Starting from a thorough investigation of mechanistic aspects of ThDP-dependent (ThDP = thiamin diphosphate) enzymes in combination with mutagenesis studies and a detailed substrate screening, new general synthetic methods have been developed based on Umpolung reactions by thiamin catalysis. A selective donor–acceptor concept was established leading to the first asymmetric cross-benzoin condensation, and a kinetic racemic resolution through C–C bond cleavage was developed. With these tools and in combination with protein engineering, we approached the synthesis of new chiral building blocks on a preparative scale. An outlook is given with respect to the potential of other ThDP-dependent enzymes as catalysts in asymmetric synthesis.

Keywords: cleavage reactions · enzyme catalysis · protein engineering · umpolung

Introduction

The first communication of the structure of thiamin diphosphate (ThDP) appeared in 1937,[1] but the application of ThDP-dependent enzymes for the production of chiral 2-hydroxyketones had been applied as long ago as 1921, when the first process based on a whole cell biotransformation was invented.[2] The process is still in use in an almost unchanged form for the production of (*R*)-phenylacetyl carbinol, the precursor of (−)-ephedrine.[2c, 3] Pyruvate decarboxylase, the enzyme responsible for the enantioselective C–C bond formation, catalyzes as a main reaction the decarboxylation of pyruvate. In a side reaction an activated acetaldehyde is ligated with benzaldehyde in a benzoin-condensation-like manner. Various other ThDP-dependent α-keto acid decarboxylases have been described as catalyzing C–C bond formation and/or cleavage.[4]

Here we want to draw attention to some concepts based on the investigation of reactions catalyzed by the enzymes pyruvate decarboxylase (PDC), benzoylformate decarboxylase (BFD), and benzaldehyde lyase (BAL), the genes of which were all cloned and the proteins overexpressed in recombinant *E. coli* strains. Extensive work has also been conducted on transketolase (TK) from different sources and recently reviewed.[5]

Pyruvate Decarboxylase (PDC) and Variants Thereof

The potential of PDC to catalyze the carboligation of acetaldehyde with both aliphatic and aromatic aldehydes was first demonstrated for the enzyme of *Saccharomyces cerevisiae* by using fermenting yeast. Studies on PDC from wheat germ and from the bacterium *Zymomonas mobilis* subsequently revealed a similar potential for PDC from various sources, also showing differences in the substrate range (for a review see reference [6]). A common principle of PDC-catalyzed carboligations is that acetaldehyde is the preferred donor substrate; however, propanal and butanal have also been described as donor aldehydes.[6] Even glyoxylate is weakly decarboxylated by PDC and the corresponding formaldehyde was shown to be transferred to acetaldehyde as an acceptor.[7] The stereocontrol of the carboligation reaction is only strict with aromatic or heterocyclic aldehydes as acceptors, while the formation of acetoins resulted in mixtures of the respective *R* and *S* enantiomer.[8]

The formation of (*R*)-1-hydroxy-1-phenyl-2-propanone [(*R*)-phenylacetylcarbinol, (*R*)-PAC] from acetaldehyde and benzaldehyde has been in the focus of research of many working groups mainly with the emphasis on optimization of

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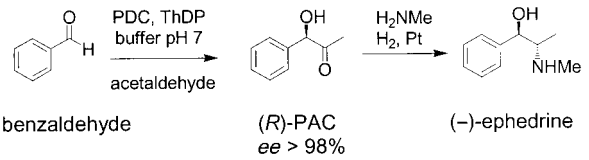


Scheme 1. Enzymatic formation of (*R*)-PAC used in the production of (−)-ephedrine.

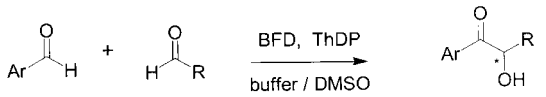
the yeast strains used for biotransformation (Scheme 1; for a review see reference [6] and Oliver et al.[9]).

Our group used a site-directed mutagenesis approach to improve the catalytic carboligase activity of PDC from *Z. mobilis* with respect to the formation of (*R*)-PAC. Replacement of a bulky tryptophane residue (W392), in the channel leading to the active center of the enzyme, by methionine or isoleucine resulted in mutant enzymes with a five- to sixfold increased carboligase activity relative to the wt-enzyme.[6]

Benzoylformate Decarboxylase (BFD) and Variants Thereof

The potential of benzoylformate decarboxylase (BFD) to catalyze C−C-bond formation was first reported by Wilcocks et al. using crude extracts of *Pseudomonas putid*a.[10] They observed the formation of (*S*)-2-hydroxy-1-phenyl-propanone ((*S*)-2-HPP) if benzoylformate was decarboxylated in the presence of acetaldehyde. Advantageously, aldehydes without a previous decarboxylation step can be used instead of the corresponding more expensive α-keto acids.[11] As depicted in Table 1, BFD is able to bind a broad range of different aromatic, heteroaromatic, and even cyclic aliphatic and conjugated olefinic aldehydes to ThDP prior to ligation to acetaldehyde.[12] Best results with respect to the enantiomeric excess (*ee*) of the resulting 2-hydroxy ketones were obtained with *meta*-substituted benzaldehydes. By using these substrates, the *ee* increased to more than 99 %, indicating that the steric demand and electronic properties of the substituent play a decisive role in both conversion rate and *ee*. *ortho*-Substituted benzaldehyde derivatives, except 2-fluorobenzaldehyde, are only poorly accepted as donor substrates by the wild-type enzyme, probably due to sterical hinderance.

Table 1. Wild-type BFD-mediated carboligation on a preparative scale.[12–14]



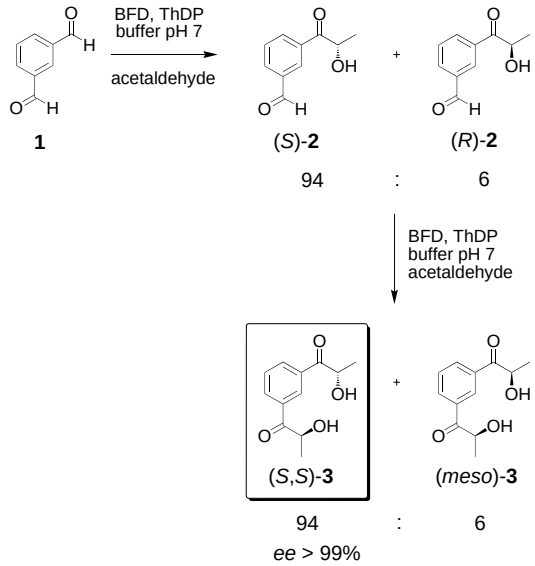
Ar	R	Yield [%]	*ee* [%]	Config.
Ph	CH_3	90	92	(*S*)
3-$MeOC_6H_4$	CH_3	97	96	(*S*)
3-*i*$PrOC_6H_4$	CH_3	91	> 99	(*S*)
3,5-di-$MeOC_6H_3$	CH_3	40	97	(*S*)
2-naphthyl	CH_3	32	88	(*S*)
Ph	Ph	70	> 99	(*R*)
2-FC_6H_4	2-FC_6H_4	68	> 99	(*R*)
4-MeC_6H_4	4-MeC_6H_4	69	> 99	(*R*)

For the first time, we demonstrated the BFD-mediated stereoselective cross-coupling of two different aliphatic substrates, cyclohexane carbaldehyde and acetaldehyde.[12] In contrast to the large variety of aromatic, olefinic, and aliphatic aldehydes that can be used as donor substrates, wild-type BFD does not tolerate a modification of the methyl group of acetaldehyde in the case of aliphatic acceptor aldehydes.

Besides acetaldehyde, BFD shows activity with aromatic and heteroaromatic aldehydes as the acceptor substrate forming enantiomerically pure (*R*)-benzoin and derivatives (Table 1).[13]

Biotransformation of hydrophobic aldehydes is possible in the presence of water-miscible organic solvents. The best results with regard to increased solubility of hydrophobic substrates together with the least loss of ligase activity of BFD were obtained by addition of DMSO.[14] In this way (*R*)-benzoin (*ee* > 99 %) was obtained in 70 % yield.[13]

Dialdehydes as substrates: Being aware that *meta*-substituted aromatic aldehydes provide the highest *ee* values in good to excellent conversion rates, we subjected isophthalaldehyde (**1**) to the BFD-catalyzed coupling reaction (Scheme 2).[15]



Scheme 2. BFD-mediated carboligation of isophthalaldehyde (**1**) and acetaldehyde yielding (*S*)-**2** and (*S*,*S*)-**3**.

It is noteworthy that the *ee* of the monoadduct (*S*)-**2** increases to some extent with a progressive in situ formation of bisadduct **3**, meaning that BFD accepts both enantiomers of **2** as substrate. Therefore, in this case it is not practicle to use BFD for kinetic racemic resolution. Nevertheless, the second reaction step proceeds completely stereospecifically within detection limits. The monoadduct (*S*)-**2** is converted to (*S*,*S*)-**3** in enantiomerically pure form, whereas the minor enantiomer (*R*)-**2** leads to *meso*-**3**. Compounds like (*S*,*S*)-**3** might become valuable intermediates for the synthesis of chiral bidendate ligands.

BFD variants L476Q and M365L-L461S, solution to the "*ortho* problem": From a mutant library generated[16] by

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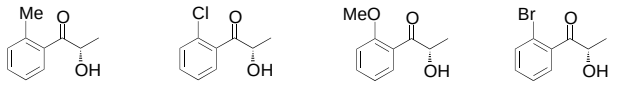
error-prone PCR, two BFD variants, L476Q and M365L-L461S, were identified as accepting *ortho*-substituted benzaldehyde derivatives as donor substrates by screening the library with 2-methylbenzaldehyde and acetaldehyde as substrates. Carboligation of these aldehydes could result in different products including 2,2′-disubstituted benzoin or 2-HPP derivatives depending on which aldehyde was accepted as donor and/or acceptor substrate. Both variants, L476Q and M365L-L461S, were shown to catalyze the formation of enantiopure (*S*)-2-hydroxy-1-(2-methylphenyl)propan-1-one ((*S*)-**4**) with excellent conversion rates. Different *ortho*-substituted benzaldehyde derivatives, such as 2-chloro-, 2-methoxy-, or 2-bromobenzaldehyde, were accepted as donor substrates by both enzymes and transformation with acetaldehyde resulted in the corresponding (*S*)-HPP derivatives **4**–**7**.[17]



(*S*)-**4** (*S*)-**5** (*S*)-**6** (*S*)-**7**

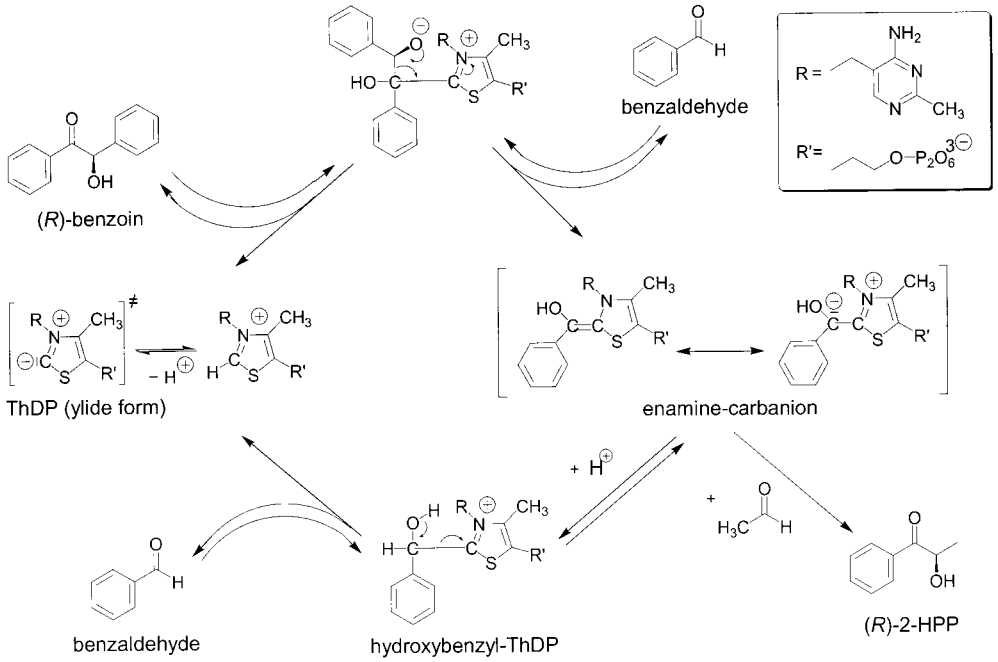
Benzaldehyde Lyase (BAL)

BAL from *Pseudomonas fluorescens* Biovar I was first reported by Gonzáles and Vicuña.[18] They showed that this strain can grow on benzoin (anisoin) as a sole carbon and energy source, due to the ability of BAL to catalyze the cleavage of the acyloin linkage of benzoin. When racemic benzoin was treated with BAL[19] in potassium phosphate buffer only a very small amount of benzaldehyde was formed. Addition of 20% DMSO as a cosolvent or alternatively 15% polyethylene glycol (PEG 400) resulted in enhanced benzaldehyde formation.[20] Only (*R*)-benzoin is converted into benzaldehyde through BAL catalysis, although complete conversion of (*R*)-benzoin was not possible under the conditions tested. Apparently, an equilibrium between cleavage and formation of (*R*)-benzoin exists during this process. (*S*)-Benzoin gave no reaction at all.[20]

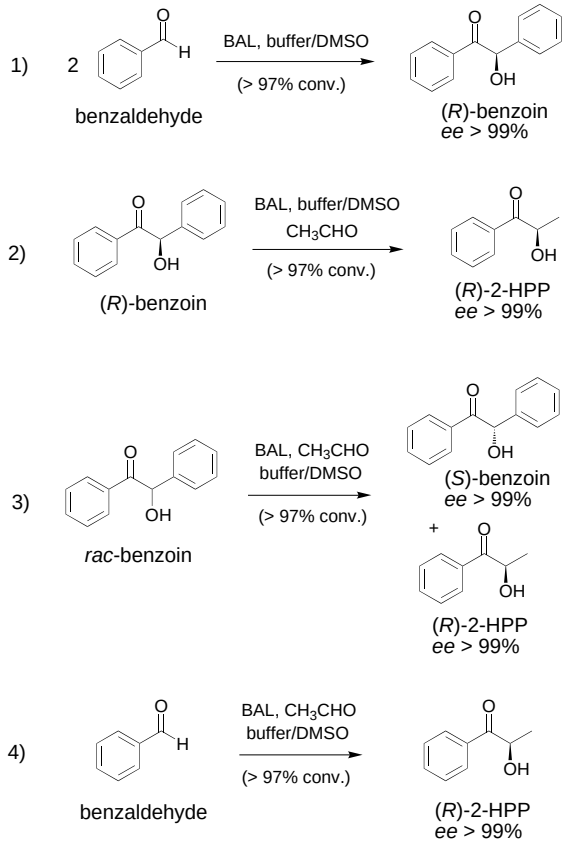
From mechanistic considerations and assuming that cleavage and formation of (*R*)-benzoin are in equilibrium (Scheme 3), BAL should also catalyze carboligation. Consequently, BAL-catalyzed acyloin condensation of benzaldehyde in aqueous buffer/DMSO mixture resulted in the almost quantitative formation of enantiomerically pure (*R*)-benzoin (Scheme 4, entry 1). The reaction was carried out on a semipreparative scale with different aromatic and heteroaromatic aldehydes.[21] From the viewpoint of the organic-preparative chemist, it is important to mention that crude cell extracts of the recombinant *E. coli* strain overexpressing the BAL gene are sufficient for catalysis; hence, purification of the enzyme is not necessary.

In contrast to BFD, BAL accepts aromatic aldehydes substituted at the *ortho*-position as well. Only a few aromatic aldehydes, such as pyridine, and 3- and 4-carbaldehyde as well as sterically exceedingly demanding aldehydes gave either very low yield or no benzoin condensation at all.[21]

Racemic resolution by C–C bond cleavage: For nonenzymatic benzoin condensations it is well established that benzoins can be used instead of aldehydes as substrates. When (*R*)-benzoin was treated with BAL in the presence of acetaldehyde (Scheme 4, entry 2) quantitative formation of enantiopure (*R*)-2-HPP occurred.[20] The same reaction starting from (*S*)-benzoin failed. Repeating this reaction with racemic benzoin afforded enantiopure (*R*)-2-HPP and (*S*)-benzoin after separation of the products by column chromatography (Scheme 4, entry 3). As expected from these results,



Scheme 3. Proposed mechanism for BAL-catalyzed acyloin formation and cleavage based on observations with other ThDP-dependent enzymes.



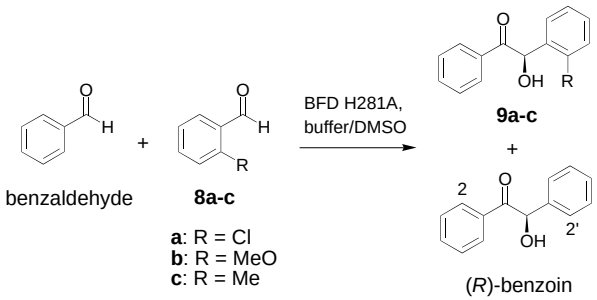
Scheme 4. Different types of reactions catalyzed by BAL.

the BAL-catalyzed reaction of benzaldehyde and acetaldehyde also gave (*R*)-2-HPP in 95% yield (Scheme 4, entry 4). Several substituted and heteroaromatic benzoins are accepted as substrate for the kinetic racemic resolution through C–C bond cleavage. The reactions work well in organic–aqueous medium, overcoming the solubility problem of lipophilic substrates and opening the way for large-scale preparations.[21]

Since there is still a lack of structural information about BAL, a structure-based discussion of the observed stereocontrol is not yet possible.

Asymmetric Cross-Benzoin Condensation

Assuming that aldehydes not accepted as donor substrates still might be suitable acceptor substrates, and vice versa, we performed a mixed enzyme–substrate screening in order to identify a biocatalytic system for the asymmetric cross-carboligation of aromatic aldehydes. For this purpose 2-chloro- (**8a**), 2-methoxy- (**8b**), and 2-methylbenzaldehyde (**8c**) were treated with different enzymes in combination with benzaldehyde (Scheme 5).[22] The three *ortho*-substituted benzaldehyde derivatives **8a–c** were chosen as putative selective acceptor substrates particularly because of their inability to form symmetrical benzoins through the wild-type BFD-catalyzed reaction, meaning that these compounds are not accepted as donor substrates by this enzyme. The BFD-mutant BFD H281A[23] was identified as a potent catalyst, resulting in the formation of the mixed benzoins 2′-methoxy-

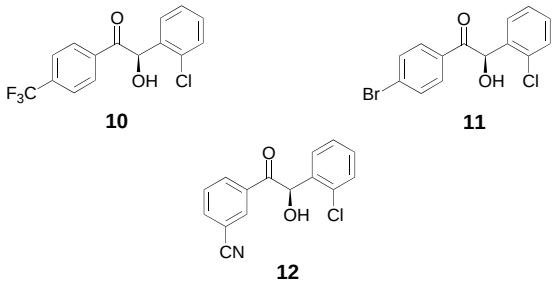


Scheme 5. Asymmetric synthesis of mixed benzoins **9a–c** by use of BFD H281A.

benzoin (**9b**) and 2′-methylbenzoin (**9c**), accompanied by (*R*)-benzoin as the major product. In the case of 2-chlorobenzaldehyde (**8a**) as acceptor substrate the unsymmetrical benzoin (*R*)-**9a** (yield 74%, *ee* > 99%) represents the major product.[22]

Remarkably, the 2,2′-disubstituted benzoin or the mixed benzoin substituted in 2-position was not generated in any of these reactions, revealing that the *ortho*-substituted benzaldehydes **8a–c** react selectively as acceptors, as expected.

Subsequently, we extended our concept to selective donor molecules. With 2-chlorobenzaldehyde as the selective acceptor a vast variety of unsymmetrical benzoins was accessible, among which **9d**, **9e** and **9f** were obtained selectively, proving that 4-(trifluoromethyl)benzaldehyde, 4-bromobenzaldehyde, and 3-cyanobenzaldehyde were selective donor substrates for BFD H281A.[22]



The selective donor–acceptor concept can be transferred to other ThDP-dependent enzymes. For example, enantiopure mixed benzoins were obtained when 2-chlorobenzaldehyde was treated with a variety of selective donor aldehydes in the presence of BAL.[22]

The selectivity is caused not only by the electronic properties of the substrates, as it was assumed in the case of nonenzymatic cross-benzoin condensation.[24] Rather, steric demands of the aldehyde substituents and interactions of these with amino acid residues in the active site of the biocatalyst, which, evidently, is different for each enzyme used, are also of significance.

Moreover, the selective donor–acceptor concept should be transferable to other fields of organic chemistry, for example, Tishchenko reactions or pinacol couplings. In this case, our work, stimulated by classic organic chemistry and carried out in the field of enzymatic synthesis, would lead us to an advanced insight into general chemical concerns.

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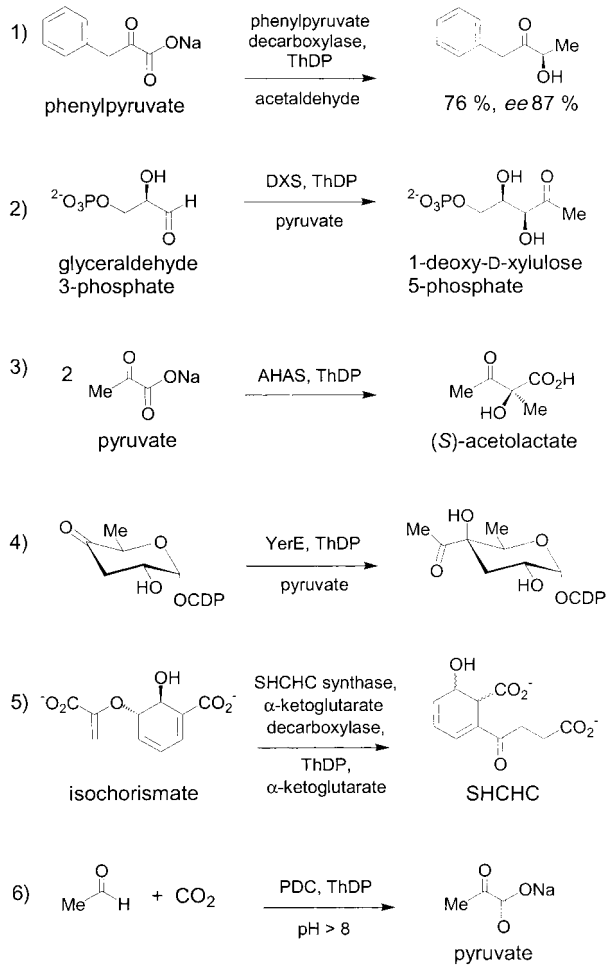
Outlook

As shown in the previous sections for the establishment of new concepts in ThDP enzyme chemistry, it is promising to elucidate the potential of biocatalysts, and, additionally, to elaborate new chemical transformations like the kinetic racemic resolution through C–C bond cleavage or the asymmetric cross-benzoin condensation.

Until now only TK,[5] PDC, BFD, BAL, and mutants thereof have been investigated systematically with regard to preparative transformations. Numerous other ThDP-dependent enzymes are capable of catalyzing different asymmetric reactions. Recently, asymmetric acyloin condensation catalyzed by phenylpyruvate decarboxylase was described by Patel et al. (Scheme 6, entry 1).[25] Indolepyruvate decarboxylases and variants thereof might be another class of enzymes



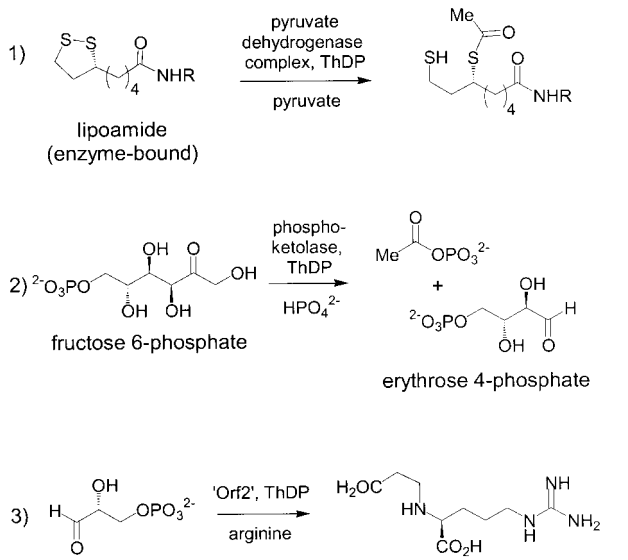
Scheme 6. Different types of reactions catalyzed by ThDP-dependent enzymes.[25–32]

capable of enlarging the substrate spectra amenable to asymmetric C–C bond formation.[26] Recombinant 1-deoxy-D-xylulose 5-phosphate synthase (DXS) has been used for the synthesis of desoxysugars in isotope-labeled or unlabeled forms (Scheme 6, entry 2).[27]

Acetohydroxy acid synthase (AHAS) has long been known for the synthesis of (*S*)-acetolactate starting from two molecules of pyruvate (Scheme 6, entry 3).[28] Most recently, Jordan and Sergienko described the same reaction catalyzed by a variant of PDC.[29] Liu and co-workers published the YerE-catalyzed ligation of activated acetaldehyde to a 4-keto-3,6-didesoxysugar, clearly demonstrating that ketones are promising acceptor substrates for ThDP-dependent enzymes, as already known from AHAS-catalyzed reactions (Scheme 6, entry 4).[30] Another highly interesting biotransformation is catalyzed by SHCHC synthase (SHCHC: 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-caboxylic acid) and α-ketoglutarate decarboxylase (Scheme 6, entry 5), readily enlarging the substrate spectra of ThDP-dependent enzymes towards C=C double bonds.[31]

Since enzymes catalyze both forward and backward reactions, the BAL-catalyzed racemic resolution of benzoins was a logical consequence (see above), and more examples for the ThDP-catalyzed racemic resolution by C–C bond cleavage should be achievable. Furthermore, carbon dioxide fixation is a likely reaction for other reversible ThDP enzymes (Scheme 6, entry 6).[32]

ThDP-catalyzed oxidative decarboxylation with formation of carbon–heteroatom bond is vastly important from the biochemical point of view. However, this has not yet been applied to non-natural substrates in preparative transformations.[33] Lipoic acid is used as an acceptor in the pyruvate dehydrogenase complex (formation of a C–S bond) finally resulting in the formation of acetyl-CoA (Scheme 7, entry 1).[34] Phosphoketolase catalyzes an irreversible ThDP-dependent phosphorolytic reaction, for example, cleaving fructose 6-phosphate in the presence of inorganic phosphate to yield erythrose 4-phosphate and acetyl phosphate (Scheme 7, entry 2).[35] Very recently, Townsend and co-workers published an example of a ThDP-dependent enzyme-catalyzed C–N bond formation (Scheme 7, entry 3).[36]

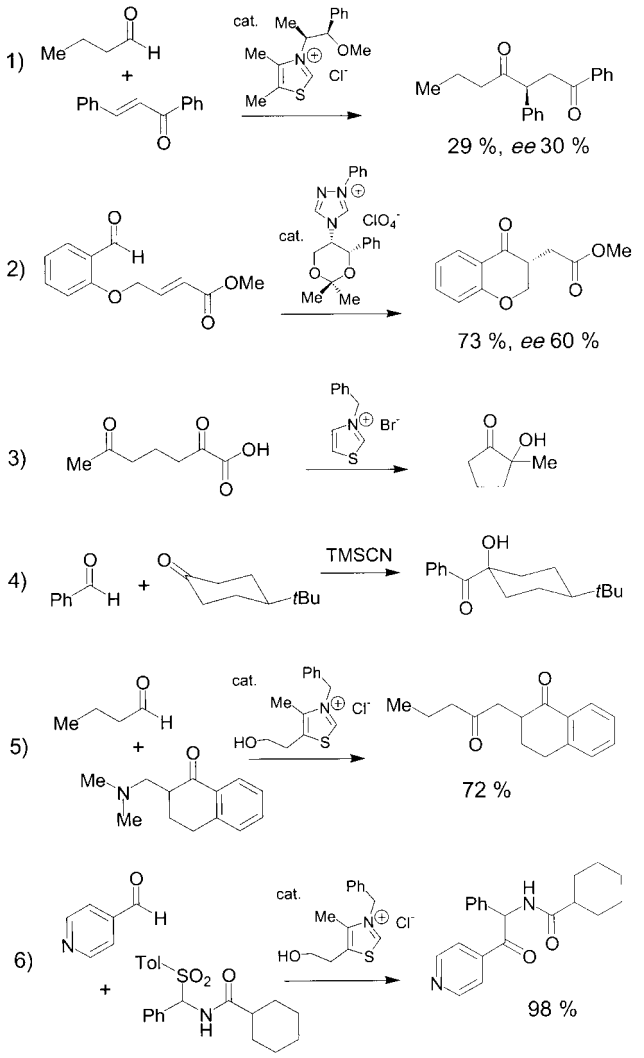


Scheme 7. Formation of carbon–heteroatomic bonds by ThDP-dependent enzyme-catalyzed reactions.[34–36]

Another very interesting aim is chain elongation through transformation of a C-1 unit (formaldehyde or equivalent),

which is very difficult to perform selectively by nonenzymatic catalysis.[37] Several ThDP-dependent enzymes (TK,[38] PDC,[7, 39] dihydroxyacetone synthase (DHAS),[40] and glyoxylate carboligase (GCL)[41]) are known to catalyze such reactions, although mostly in a nonasymmetric manner.

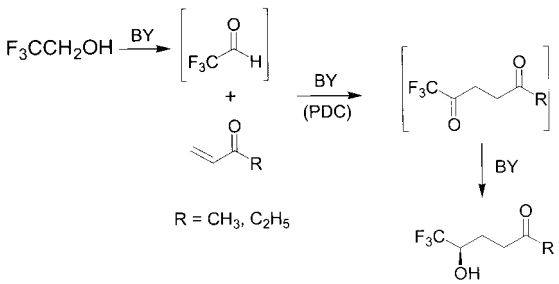
Other putative acceptor substrates known from nonenzymatic benzoin condensations and related reactions are Michael acceptors for the intermolecular (Scheme 8, entry 1)[42] and intramolecular Stetter reaction (entry 2),[43]



Scheme 8. Different types of reactions catalyzed by thiazolium- or triazolium-derivatives or cyanide.[42–47]

diketosubstrates for ring-closing reactions (entry 3),[44] ketones (entry 4),[45] Mannich bases (entry 5),[46] and imines (entry 6).[47] Remarkably, in the last case the corresponding benzoins are not observed and do not serve as substrates either.[47d] It will be very interesting to see whether this reaction can be performed in an asymmetric manner, either with chemical catalysts or enzymes.[48]

The enzyme-catalyzed Stetter reaction has already been described in the literature, although the cited whole-cell biotransformation has not been elucidated in detail (Scheme 9).[49]



Scheme 9. Enzyme-catalyzed Stetter reaction (proposed reaction pathway).[49]

Conclusion

The detailed investigation of the ThDP-dependent enzymes PDC, BFD, and BAL by using techniques of substrate *and* protein engineering resulted in new concepts in chemoenzymatic synthesis, such as kinetic resolution through C–C bond cleavage and asymmetric cross-benzoin condensation. The described selective donor–acceptor concept should also be transferable to other types of enzymatic and nonenzymatic cross-coupling reactions. The racemic resolution by C–C bond cleavage established for BAL-catalyzed reactions might serve as a prototype for other ThDP-dependent enzyme-catalyzed resolutions. Moreover, we propose that a detailed investigation of different ThDP-dependent enzymes with the focus on new transformations, in combination with appropriate screening methods, will open new perspectives in catalytic asymmetric synthesis and in the biotechnology industry.

Hence, for chemists it is not only important to understand the tools nature uses, but they should also be aware of biocatalysts as valuable tools themselves. Biochemists/enzymologists can also profit from knowledge about established chemical transformations like the Diels–Alder reaction[50] or, as shown here, the Stetter and related reactions.

Acknowledgement

We are indebted to our talented and enthusiastic co-workers whose names are listed in the references. We thank Prof. Georg Sprenger and Dr. Andreas Liese for a fruitful collaboration in the scope of SFB 380, and Prof. Ayhan S. Demir and Dr. Michael McLeish for helpful discussions. The work by our groups summarized in this paper has been generously supported by the Deutsche Forschungsgemeinschaft (SFB 380).

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