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## Catalytic promiscuity of a proline-based tautomerase

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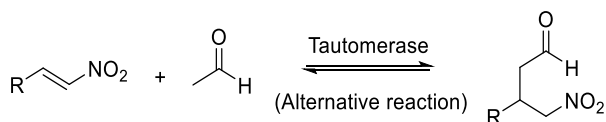
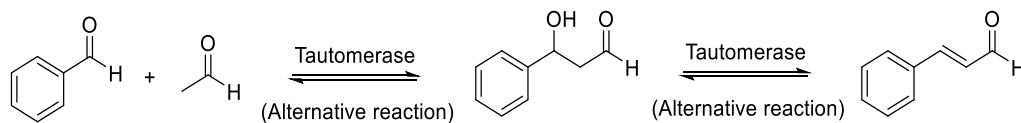
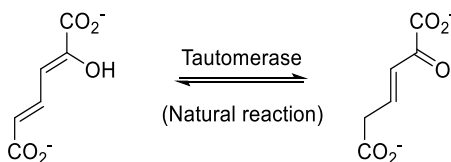
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# CHAPTER 1

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## RECENT DEVELOPMENTS IN ENZYME PROMISCUITY FOR CARBON-CARBON BOND-FORMING REACTIONS

Yufeng Miao, Mehran Rahimi, Edzard M. Geertsema and Gerrit J. Poelarends, *Curr. Opin. Chem. Biol.* 2015, **25**, 115-123.



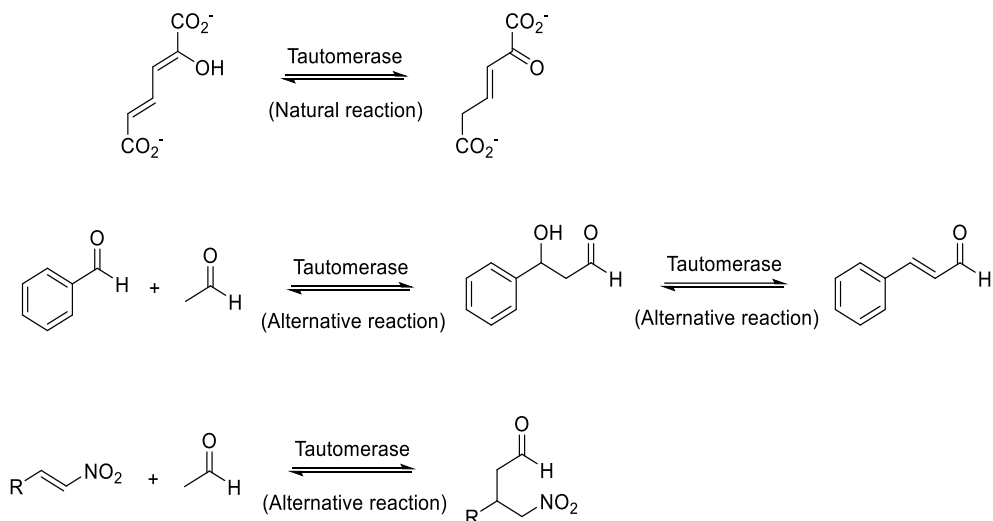
Numerous enzymes have been found to catalyze additional and completely different types of reactions relative to the natural activity they evolved for. This phenomenon, called catalytic promiscuity, has proven to be a fruitful guide for the development of novel biocatalysts for organic synthesis purposes. As such, enzymes have been identified with promiscuous catalytic activity for, one or more, eminent types of carbon-carbon bond-forming reactions like aldol couplings, Michael(-type) additions, Mannich reactions, Henry reactions, and Knoevenagel condensations. This review focuses on enzymes that promiscuously catalyze these reaction types and exhibit high enantioselectivities (in case chiral products are obtained).

### 1.1 Introduction

Documented examples of enzymes that catalyze, one or more, mainstream carbon-carbon bond-forming reactions such as Michael(-type) additions, Mannich reactions, Henry reactions, or Knoevenagel condensations as their natural activity are extremely rare while aldolases, which catalyze aldol couplings as their natural activity, often exhibit limited substrate acceptance. An applied strategy to address these issues is the exploration of enzyme promiscuity,<sup>[1-4]</sup> which can be defined as 'enzyme activities other than the activity for which an enzyme evolved and that are not part of the organism's physiology'.<sup>[5]</sup> For example, the enzyme 4-oxalocrotonate tautomerase (4-OT) naturally catalyzes an enol-keto tautomerization step as part of a catabolic pathway for aromatic hydrocarbons in *Pseudomonas putida* mt-2, but also promiscuously catalyzes aldol condensation and Michael-type addition reactions (Scheme 1.1).<sup>[4]</sup> Recently, enzymatic methods for carbon-carbon bond formation reactions and their applications have been reviewed.<sup>[6-10]</sup> Yet, examples of enzymes with promiscuous carbon-carbon bond formation activities were only briefly mentioned. In this review, we highlight recent advances in enzyme promiscuity for a number of important carbon-carbon bond-forming reactions with a focus on research contributions that report:

1. enantioselective enzyme catalysis (in case chiral products are obtained) since enantioselective carbon-carbon bond formation is a most important and challenging aspect of organic synthesis.
2. a methodology that can be carried out at (semi-)preparative scale.
3. proper control experiments to ensure that product formation is effected by the anticipated enzyme and not by a contaminating protein or by a non-enzymatic 'background' reaction.

The following paragraphs have been organized based on reaction types (aldol couplings and condensations, Michael(-type) additions, Mannich reactions, Henry reactions, and Knoevenagel condensations) after which a summary, perspectives and conclusions are presented.



**Scheme 1.1:** An enol-keto tautomerase also catalyzes C-C bond-forming aldol condensation and Michael-type addition reactions.

## 1.2 Aldol couplings and condensations

An aldol coupling generally refers to the nucleophilic addition of the enolate of a ketone, or an aldehyde, to another carbonyl compound to produce a  $\beta$ -hydroxyl aldehyde or ketone. The formed aldol coupling product may undergo dehydration to form an  $\alpha,\beta$ -unsaturated carbonyl compound. The combined process of an aldol coupling and subsequent dehydration is called an aldol condensation.

Since Berglund and coworkers reported the promiscuous catalytic activity of lipase from *Candida antarctica* (CAL-B) for the aldol coupling between linear aldehydes in 2003,<sup>[11]</sup> CAL-B and several other lipases have been intensively studied for their promiscuous aldolase activities. However, it took until 2008 before the first asymmetric lipase-catalyzed *asymmetric* aldol coupling was reported.<sup>[12]</sup>

Yu *et al.* described the aldol addition of acetone (**2**,  $\text{R}^2 = \text{Me}$ ) to 4-nitrobenzaldehyde (**1**,  $\text{R}^1 = 4\text{-NO}_2$ ) catalyzed by lipase from porcine pancreas (PPL) (Table 1.1).<sup>[12]</sup> Conditions could be tuned so that aldol coupling adduct **3** ( $\text{R}^1 = 4\text{-NO}_2$ ,  $\text{R}^2 = \text{Me}$ ) was either obtained with 96% yield and 15% enantiomeric excess (*ee*) or with 12% yield and 44% *ee* (Table 1.1, entries 1 and 2). This trend of an increase of yield going hand in hand with a decrease of enantioselectivity (and vice versa), either by changing reaction conditions or by offering different substrate derivatives, is observed within the majority of methodologies for promiscuous enzyme-catalyzed aldol couplings that we describe in this section (one of the few exceptions is represented by PPL-catalyzed aldol couplings of **1** with **5** (vide infra)). We have chosen to enlist the examples of a collection of methodologies for

promiscuous enzymatic aldol couplings that feature the highest enantiomeric excesses (see Table 1.1).

Yu *et al.* reported that also the protease pepsin catalyzes the asymmetric aldol coupling of various substituted benzaldehydes **1** and ketones **2**.<sup>[13]</sup> The highest enantiomeric excess was observed with substrates 4-nitrobenzaldehyde **1** ( $R^1 = 4\text{-NO}_2$ ) and acetone (**2**,  $R^2 = \text{Me}$ ) giving product **3** ( $R^1 = 4\text{-NO}_2$ ,  $R^2 = \text{Me}$ ) with 45% *ee* (Table 1.1, entry 3). Recently, the same investigators presented a very interesting example of combining the natural esterase activity with the promiscuous aldolase activity of a lipase to perform aldol condensation reactions between various aromatic aldehydes (**1**) and *in situ* generated acetaldehyde (**2**,  $R^2 = \text{H}$ ) (Table 1.1, entry 4).<sup>[14]</sup> During this one-pot tandem process, the hydrolysis of substrate vinyl acetate to give **2** ( $R^2 = \text{H}$ ) as well as the subsequent aldol condensation of **2** ( $R^2 = \text{H}$ ) with the aldehyde substrate (**1**) are catalyzed by lipase from *Mucor miehei* (MML). The highest yield of dehydrated product **4** (78%) was obtained with acceptor 4-nitrobenzaldehyde (**1**,  $R^1 = 4\text{-NO}_2$ ). The authors do not mention if they observed aldol coupling intermediates **3** nor whether the dehydration of **3** into **4** is MML-catalyzed or not.

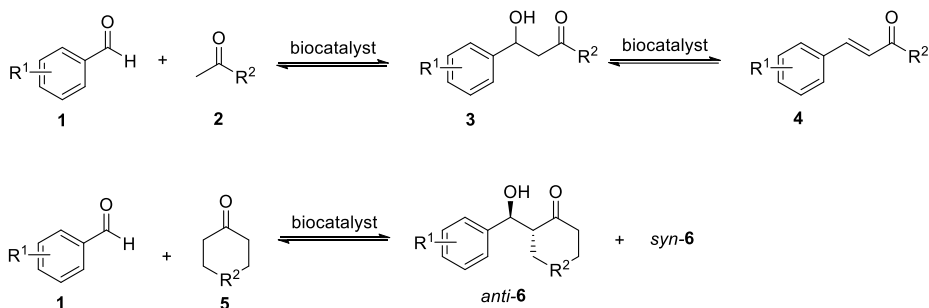
Another intriguing example of a one-pot enzymatic tandem reaction was presented by Poelarends and co-workers.<sup>[15,16]</sup> The enzyme 4-oxalocrotonate tautomerase (4-OT) promiscuously catalyzes the aldol coupling of acetaldehyde (**2**,  $R^2 = \text{H}$ ) with benzaldehyde (**1**,  $R^1 = \text{H}$ ), to give **3** ( $R^1 = R^2 = \text{H}$ ), and the subsequent dehydration (of **3**) yielding **4** ( $R^1 = R^2 = \text{H}$ ). Intermediate **3** was not observed (<sup>1</sup>H NMR) in the course of the reaction. However, offering chemically synthesized **3** ( $R^1 = R^2 = \text{H}$ ) to 4-OT revealed that the enzyme indeed catalyzes the dehydration of **3** into **4** ( $R^1 = R^2 = \text{H}$ ).<sup>[16]</sup> The promiscuous aldolase activity of 4-OT proceeds via an anticipated catalytic mechanism. The catalytic N-terminal proline (Pro1) residue of 4-OT acts as a nucleophile and forms an enamine intermediate with **2** ( $R^2 = \text{H}$ ) which subsequently reacts with benzaldehyde **1** ( $R^1 = \text{H}$ ). The rather low-level aldolase activity of wild-type 4-OT was improved by 600-fold in terms of catalytic efficiency ( $k_{\text{cat}}/K_{\text{m}}$ ) by a single point mutation (F50A) (Table 1.1, entry 5).<sup>[16]</sup> The enantioselectivity of 4-OT, and the just-mentioned lipase MML, for aldol couplings of substrates **1** and **2** could not be ascertained since dehydrated products **4** are non-chiral and because chiral intermediates **3** were not observed during reaction, let alone examined on enantiomeric excess.

Guan and coworkers have extensively examined promiscuous catalysis of aldol couplings of substituted benzaldehydes (**1**) with cyclic ketones (**5**) by various types of enzymes including lipase PPL (II),<sup>[17]</sup> nuclease p1,<sup>[18]</sup> and proteases such as alkaline protease from *Bacillus licheniformis* (BLAP),<sup>[19]</sup> chymopapain,<sup>[20]</sup> acidic protease from *Aspergillus usamii* (AUAP),<sup>[21]</sup> protease from *Aspergillus melleus* (AMP),<sup>[22]</sup> trypsin,<sup>[23]</sup> and ficin<sup>[24]</sup> (Table 1.1, entries 6-15). Products **6** were obtained with *anti/syn* ratios ranging from 53/47 (PPL (II):  $R^1 = 2\text{-NO}_2$ ,  $R^2 = \text{N-Boc}$ , Table 1.1, entry 6)<sup>[17]</sup> to 92/8 (AMP:  $R^1 = 2\text{-NO}_2$ ,  $R^2 = \text{CH}_2$ , Table 1.1, entry 13)<sup>[22]</sup> while excellent enantiomeric excesses of 99% were established with BLAP ( $R^1 = 4\text{-Me}$ ,  $R^2 = \text{CH}_2$ , Table 1.1, entry 9)<sup>[19]</sup> and nuclease p1 ( $R^1 = 4\text{-Me}$ ,  $R^2 = \text{CH}_2$ , Table 1.1, entry 8).<sup>[18]</sup> Yu *et al.* found that also lipase BPL catalyzes

the aldol coupling of **1** with **5** ( $R^1 = 3\text{-NO}_2$ ,  $R^2 = \text{CH}_2$ , Table 1.1, entry 16) ( $dr = 72/28$ ,  $ee = 66\%$ ).<sup>[25]</sup> It should be emphasized once more that we gave the example of each methodology that features the highest enantiomeric excess. Within most methodologies higher product yields are reported, however, in the majority of cases at the expense of lower enantiomeric excesses (for example, compare entries **6** with **7** and **11** with **12** in Table 1.1). An exception to this general observation is represented by the PPL-methodology for the aldol coupling of **1** with **5** developed by Yu *et al.* (entries 17 and 18).<sup>[26]</sup> In this specific case, high product yields of **6** are accompanied with excellent  $dr$ 's and  $ee$ 's (**6**:  $R^1 = 4\text{-F}$ ,  $R^2 = \text{CH}_2$ : yield = 75%;  $dr = 88:12$ ;  $ee = 90\%$  and **6**:  $R^1 = 4\text{-Br}$ ,  $R^2 = \text{CH}_2$ : yield = 99%;  $dr = 88:12$ ;  $ee = 87\%$ ).

## Chapter 1. Recent developments in enzyme promiscuity for C-C bond-forming reactions

**Table 1.1:** Substrates, biocatalysts, solvents, product structures, product yields, diastereomeric ratios (*dr*) and enantiomeric excesses (*ee*) regarding enzyme promiscuity for aldol couplings and condensations.

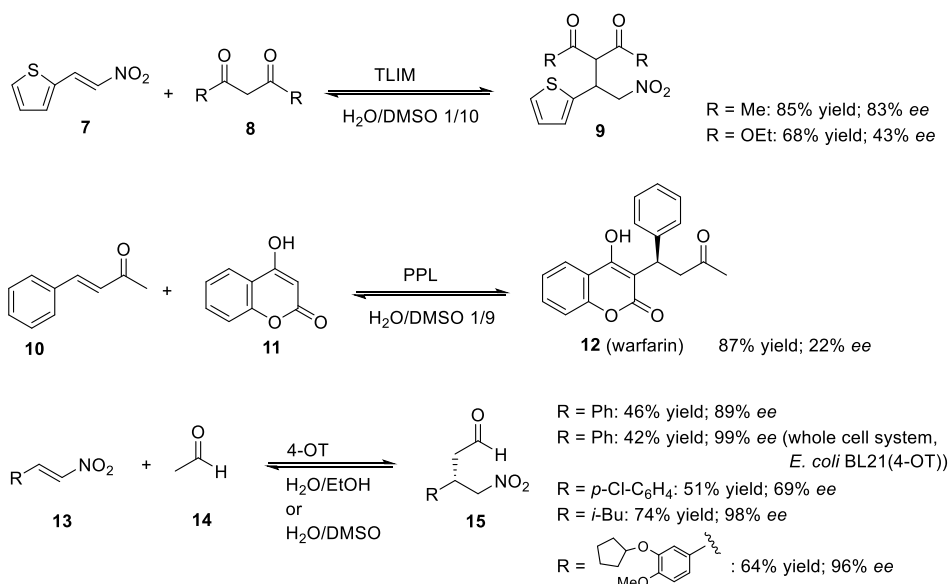


entry	substrates			biocatalyst	solvent	product	yield (%)	<i>dr anti/syn</i>	<i>ee</i> (%)	ref
		R <sup>1</sup>	R <sup>2</sup>							
1	1 and 2	4-NO <sub>2</sub>	Me	PPL	H <sub>2</sub> O	3	12	na	44	[12]
2	1 and 2	4-NO <sub>2</sub>	Me	PPL	H <sub>2</sub> O	3	96	na	15	[12]
3	1 and 2	4-NO <sub>2</sub>	Me	pepsin	H <sub>2</sub> O	3	89	na	45	[13]
4	1 and 2	4-NO <sub>2</sub>	H	MML	H <sub>2</sub> O	4	78	na	na	[14•]
5	1 and 2	H	H	4-OT F50A	H <sub>2</sub> O	4	50	na	na	[16•]
6	1 and 5	2-NO <sub>2</sub>	<i>N</i> -Boc	PPL (II)	MeCN/ H <sub>2</sub> O	6	36	53:47	87	[17]
7	1 and 5	4-NO <sub>2</sub>	O	PPL (II)	MeCN/ H <sub>2</sub> O	6	56	38:62	46	[17]
8	1 and 5	4-Me	CH <sub>2</sub>	nuclease p1	H <sub>2</sub> O	6	25	80:20	99	[18]
9	1 and 5	4-Me	CH <sub>2</sub>	BLAP	DMSO/H <sub>2</sub> O	6	28	70:30	99	[19]
10	1 and 5	4-Me	CH <sub>2</sub>	chymopapain	MeCN/ H <sub>2</sub> O	6	23	63:37	96	[20]
11	1 and 5	3-Cl	CH <sub>2</sub>	AUAP	MeCN/ H <sub>2</sub> O	6	29	92:8	88	[21]
12	1 and 5	4-NO <sub>2</sub>	CH <sub>2</sub>	AUAP	MeCN/ H <sub>2</sub> O	6	63	83:17	82	[21]
13	1 and 5	2-NO <sub>2</sub>	CH <sub>2</sub>	AMP	MeCN/ H <sub>2</sub> O	6	52	92:8	91	[22]
14	1 and 5	4-CF <sub>3</sub>	CH <sub>2</sub>	trypsin	H <sub>2</sub> O	6	34	59:41	65	[23]
15	1 and 5	3-NO <sub>2</sub>	CH <sub>2</sub>	ficin	MeCN/ H <sub>2</sub> O	6	39	86:14	81	[24]
16	1 and 5	3-NO <sub>2</sub>	CH <sub>2</sub>	BPL	H <sub>2</sub> O	6	91	72:28	66	[25]
17	1 and 5	4-F	CH <sub>2</sub>	PPL	5 and H <sub>2</sub> O	6	75	88:12	90	[26•]
18	1 and 5	4-Br	CH <sub>2</sub>	PPL	5 and H <sub>2</sub> O	6	99	88:12	87	[26•]

na = not applicable

### 1.3 Michael(-type) additions

The name 'Michael addition' was originally given to carbon-carbon bond-forming addition reactions of enolate-type donors to  $\alpha,\beta$ -unsaturated carbonyl acceptors. Therefore, any similar type of addition reaction but employing a different type of donor and/or acceptor should be regarded as a 'Michael-type addition'. Examples of enzyme-catalyzed Michael(-type) additions for carbon-carbon bond formation are rare and the majority described in the literature involves catalytic promiscuity of an enzyme. [27]



**Scheme 1.2:** Enzyme promiscuity for asymmetric carbon-carbon bond-forming Michael(-type) additions.

One of the first planned searches for promiscuous carbon-carbon bond-forming Michael(-type) reactions was documented in 1996 and involves the addition of 1,3-dicarbonyl donors to nitroolefin acceptors catalyzed by lipase from *Pseudomonas* species (PSL). [28] Later, Berglund and co-workers discovered that *Pseudozyma antarctica* lipase B (PalB, formally known as CALB) catalyzes the Michael addition of 1,3-dicarbonyls to  $\alpha,\beta$ -unsaturated carbonyl acceptors. [29,30] Although both methodologies involve formation of chiral products, enantiomeric excesses were not reported.

Guan and coworkers reported one of the first examples of asymmetric enzyme-catalyzed carbon-carbon bond-forming Michael(-type) additions. [31] Their methodology involves immobilized lipase from *Thermomyces langinosus* (Lipozyme TLIM) and includes a wide range of 1,3-dicarbonyls and ketones as donors, and various nitroolefins



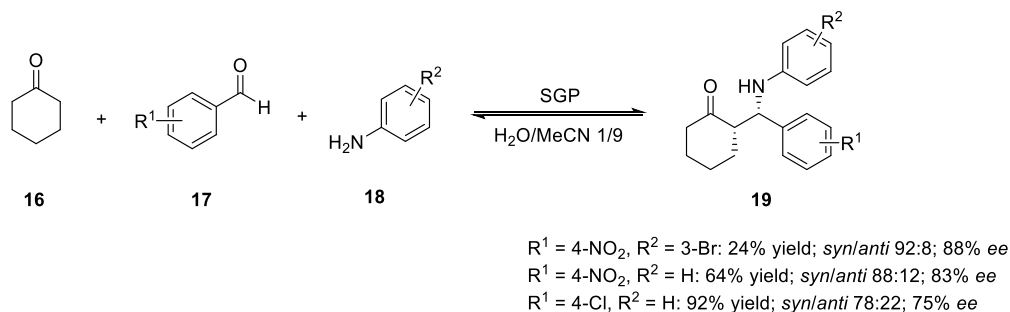
and cyclohexenones as acceptors. In terms of enantioselectivities, best results were achieved with (*E*)-2-(2-nitrovinyl)thiophene (**7**) as acceptor and 2,4-pentadione (**8**, R = Me) and diethyl malonate (**8**, R = OEt) as donors leading to enantiomeric excesses of Michael addition products **9** of 83 and 43%, respectively (Scheme 1.2). The same research group presented an elegant biocatalytic approach for the synthesis of the anti-coagulant warfarin (**12**) and derivatives.<sup>[32]</sup> A lipase PPL-mediated Michael addition of 4-hydroxycoumarin (**11**) to benzylideneacetone (**10**) furnished warfarin (**12**) with 22% *ee* (Scheme 1.2).

Recently, Poelarends and co-workers established enzyme-catalyzed asymmetric Michael-type additions producing  $\gamma$ -nitroaldehydes **15** (R = Ph, *p*-Cl-C<sub>6</sub>H<sub>4</sub>, *i*-Bu, 3-*c*-PentO-4-MeO-C<sub>6</sub>H<sub>3</sub>) with excellent enantiomeric excesses of up to 98% (Scheme 1.2).<sup>[33–36]</sup> This novel biocatalytic methodology employs the proline-based tautomerase 4-OT (see also paragraph 'Aldol couplings and condensations') which accepts a wide range of linear aldehyde donors, including acetaldehyde (**14**), and a series of aromatic and aliphatic nitroolefin (**13**) acceptors as substrates. The  $\gamma$ -nitroaldehyde products (**15**) can be readily converted into valuable GABA-based pharmaceuticals such as phenibut, baclofen, pregabalin, and rolipram (see<sup>[35]</sup> for relevant references). Whereas the other examples reviewed in this paragraph were discovered by screening a collection of robust, commercially available enzymes in the presence of appropriate substrates, the 4-OT methodology represents a designed strategy for promiscuous enzyme-catalyzed carbon-carbon bond-forming Michael(-type) additions. The N-terminal Pro-1 residue of 4-OT forms an envisaged nucleophilic enamine intermediate with acetaldehyde (**14**) which subsequently adds to the double bond of the nitroolefin creating the new carbon-carbon bond. Finally, hydrolysis releases the product from the enzyme. Interestingly, Nikodinov-Runic *et al.* tested 4-OT in a whole cell system (*E. coli* BL21(4-OT)) for the Michael-type addition of acetaldehyde (**14**) to several  $\beta$ -nitrostyrene derivatives and established enantiomeric excesses of up to 99% (**15**: R = Ph).<sup>[37]</sup>

### 1.4 Mannich reactions

A direct Mannich reaction is a three component reaction during which an aldehyde and amine form an imine that functions as an acceptor for a subsequent carbon-carbon bond-forming addition of an enolate of a carbonyl substrate. To the best of our knowledge there are no documented examples of enzymes that catalyze Mannich reactions as their natural activity. There are a few recent reports however on enzymes that exhibit promiscuous Mannich reaction activity.<sup>[38–41]</sup> All these examples feature an imine that is formed from an aromatic amine (**18**) and a benzaldehyde type substrate (**17**) while the enolate is generated from acetone or cyclohexanone (**16**), or a derivative thereof. Biocatalysts that promiscuously catalyze Mannich reactions include lipase from *Mucor miehei* (MML),<sup>[38]</sup> lipase from *Candida rugosa* (CRL),<sup>[39]</sup> trypsin from hog pancreas,<sup>[40]</sup> and protease type XIV from *Streptomyces griseus* (SGP).<sup>[41]</sup> Chiral products are obtained with all these biocatalytic systems. However, only the SGP-methodology, established by Guan *et al.*,<sup>[41]</sup>

was reported to effect enantioenriched products and herewith represents the first biocatalytic *asymmetric* Mannich reaction (Scheme 1.3).



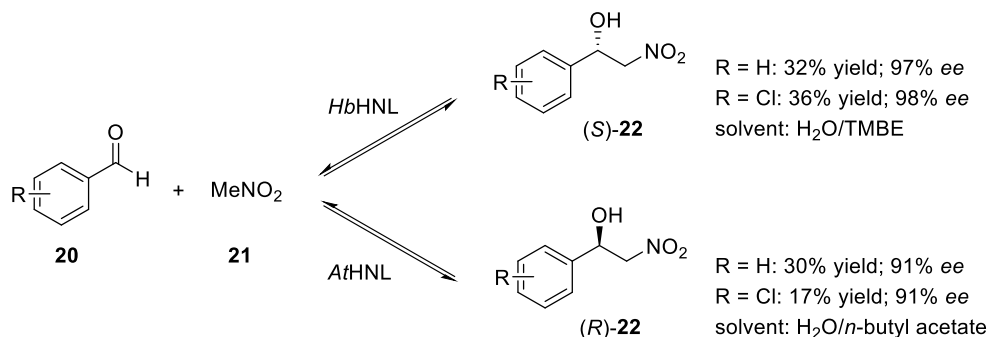
**Scheme 1.3:** Enzyme promiscuity for asymmetric Mannich reactions.

Substrates cyclohexanone (**16**), 4-nitrobenzaldehyde (**17**,  $\text{R}^1 = 4\text{-NO}_2$ ), and 3-bromoaniline (**18**,  $\text{R}^2 = 3\text{-Br}$ ) were converted by SGP to give product **19** with a diastereomeric ratio of 92/8 (*syn/anti*) and enantiomeric excess of 88%. Variation of substituents of substrates **17** and **18** effected higher yields but lower stereoselectivities (Scheme 1.3).

## 1.5 Henry reactions

Hydroxynitrile lyases (HNLs) constitute a family of enzymes that catalyze the reversible decomposition of cyanohydrins into the corresponding aldehyde, or ketone, and hydrogen cyanide (HCN). Purkarthofer *et al.* reported that HNL from *Hevea brasiliensis* (*HbHNL*) exhibits promiscuous Henry reaction activity and catalyzes the addition of nitromethane (**21**) to various types of aldehydes (**20**) yielding (*S*)- $\beta$ -nitroalcohols (**22**) (Scheme 1.4).<sup>[42]</sup> Intriguingly, a few years later, Asano *et al.* described that HNL from *Arabidopsis thaliana* (*AtHNL*) promiscuously catalyzes the identical type of reaction but yielding products **22** with the opposite (*R*)-configuration (*i.e.* (*R*)- $\beta$ -nitroalcohols) (Scheme 1.4).<sup>[43]</sup> For example, substrates **20** ( $\text{R} = \text{H}$ ) and **21** were converted into (*S*)-**22** ( $\text{R} = \text{H}$ , *ee* 97%) in the presence of *HbHNL* while *AtHNL* effected formation of (*R*)-**22** ( $\text{R} = \text{H}$ , *ee* 91%). The identical observation was made with 3-Cl-benzaldehyde (**20**,  $\text{R} = 3\text{-Cl}$ ) and **21** as substrates.

A number of other enzymes, such as transglutaminase (TGase)<sup>[44]</sup> and Amano acylase (AA) from *Aspergillus oryzae*,<sup>[45]</sup> and proteins such as gelatin and collagen,<sup>[46]</sup> have been described to promiscuously catalyze Henry reactions. However, none of them delivers products with enantiomeric excess.



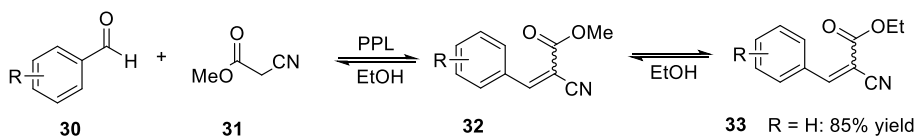
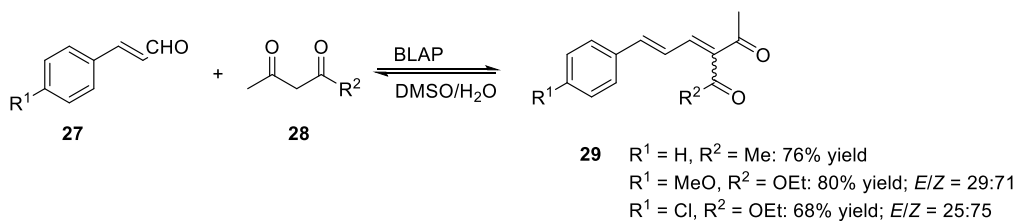
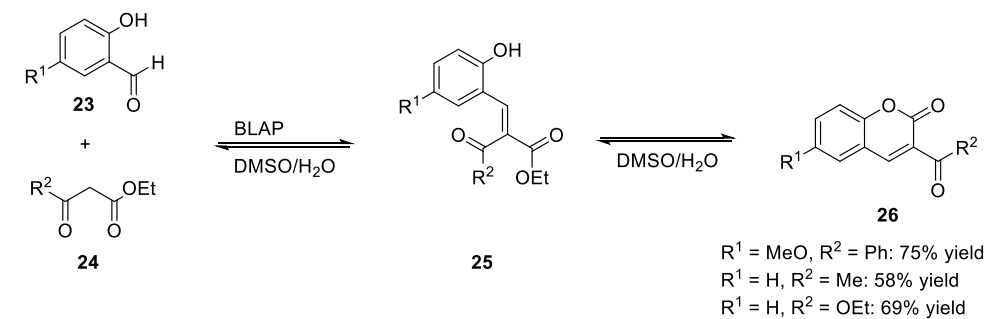
Scheme 1.4: Enzyme promiscuity for asymmetric Henry reactions.

## 1.6 Knoevenagel condensations

The Knoevenagel condensation is another example of a mainstream carbon-carbon bond-forming reaction for which no enzyme with natural activity has been reported yet. Recently though, a number of enzymes with promiscuous Knoevenagel condensation activity has been described. Guan and coworkers developed a biocatalytic two-step tandem process, including a Knoevenagel condensation and intramolecular transesterification, for the synthesis of 2H-1-benzopyran-2-ones **26**<sup>[47]</sup> (Scheme 1.5) which are important structural motifs regarding pharmaceuticals such as the anticoagulant warfarin.<sup>[32]</sup> The protease BLAP catalyzes the conversion of diverse salicylaldehydes **23** and 1,3-dicarbonyl compounds **24** into products **26** in overall yields of up to 75%. Control experiments clearly demonstrated that the first step, the Knoevenagel condensation giving intermediate **25**, is BLAP-catalyzed. The evidence that BLAP also catalyzes the subsequent intramolecular transesterification into **26** is less compelling. The same authors showed that BLAP as well catalyzes the Knoevenagel condensation of cinnamaldehyde **27**, and derivatives, with various 1,3-dicarbonyl compounds **28** furnishing adducts **29** in yields of up to 80% and *E/Z* ratios of 25:75<sup>[48]</sup> (Scheme 1.5).

Lai *et al.* discovered that lipase PPL is able to catalyze a Knoevenagel condensation between benzaldehyde (**30**, R = H), and derivatives hereof, and methyl cyanoacetate (**31**) to give adducts **32** in good overall yields<sup>[49]</sup> (Scheme 1.5). No comments are made on the *E/Z* ratios with which products **32** are obtained nor on which isomer is obtained in excess. The authors claim that the observed subsequent transesterification of **32** into **33** is also PPL-catalyzed for which, however, no convincing evidence is provided.

## 1.6. Knoevenagel condensations



Scheme 1.5: Enzyme promiscuity for Knoevenagel condensations.

## 1.7 Summary, perspectives, and concluding remarks

In this review, we have summarized recent examples of catalytic promiscuity for five important classes of carbon-carbon bond-forming reactions: aldol couplings and condensations, Michael(-type) additions, Mannich reactions, Henry reactions, and Knoevenagel condensations. For developing biocatalytic methodologies for the last four types of reactions one has to rely mainly on enzyme promiscuity as enzymes that catalyze, one or more of, these reactions naturally are rare. Most of the methods we describe in this review were discovered by screening a library of commercially available biocatalysts such as lipases, proteases, nucleases, acylases, and transglutaminases, for desired promiscuous activities with a series of substrates. The 4-OT-methodology is an exception as its aldol coupling and Michael(-type) addition activities take place via an envisioned catalytic mechanism.<sup>[15,33]</sup>

The majority of papers on enzymatic promiscuity for carbon-carbon bond-forming reactions that yield chiral products report low, or no, enantioselectivities despite the fact that enzymes provide a natural chiral environment for asymmetric catalysis. With this review therefore, we have focused on those contributions that describe formation of enantioenriched products (in case chiral products are obtained).

Numerous biocatalysts with promiscuous, stereoselective aldolase activity have been identified some of which provide aldol adducts with excellent enantiomeric excesses of up to 99% (Scheme 1.1, Table 1.1). High enantiomeric excesses of products are usually accompanied with low yields. Improvement of product yields may be achieved by offering different substrate derivatives or by changing reaction conditions,<sup>[50]</sup> but often show concomitant decrease of enantiomeric excess. This trend is also observed within the first methodology for enantioselective enzyme-catalyzed Mannich reactions which provides products with good enantiomeric excesses of up to 88% (Scheme 1.3). An interesting phenomenon is observed for HNL-catalyzed Henry reactions. *HbHNL* catalyzes formation of  $\beta$ -nitroalcohols with (*S*)-configuration while *AtHNL* gives the identical products with (*R*)-configuration (Scheme 1.4). High enantiomeric excesses were established (*HbHNL*: 98% *ee*; *AtHNL*: 91% *ee*) while product yields are moderate. Biocatalysts TLIM, PPL, and 4-OT have been reported to exhibit promiscuous activity for enantioselective carbon-carbon bond-forming Michael(-type) additions (Scheme 1.2). Of these three enzymes, best enantioselectivity is exerted by the tautomerase 4-OT as it facilitates formation of valuable  $\gamma$ -nitroaldehydes with enantiomeric excesses of up to 99% and in good yields. A number of interesting and useful methodologies have been developed for biocatalytic Knoevenagel condensations which, by definition, do not involve the creation of a new chiral center (Scheme 1.5). The protease BLAP and lipase PPL give Knoevenagel products with good yields of up to 85%.

All in all, significant advances have been made in the area of promiscuous enzyme-catalyzed carbon-carbon bond-forming reactions during the last five years. At the same time, the number of enzymatic methodologies for asymmetric catalysis of mainstream carbon-carbon bond-forming reactions is still limited. Therefore challenges for the near future are 1) to translate currently available biocatalytic methodologies, which are of-

ten based on low-level promiscuous carbon-carbon bond-forming activities, into practical, efficient, and highly stereoselective organic synthesis procedures; and 2) to use the understanding of reaction mechanisms to systematically screen for new promiscuous activities in existing enzymes, and exploiting this promiscuity as starting point to develop novel enantioselective biocatalytic methods for important carbon-carbon bond-forming reactions.<sup>[51]</sup> In general, the approach of exploiting catalytic promiscuity as starting point to create tailor-made biocatalysts may support a new and exciting area in protein engineering research, and may be the key to more application of biocatalysis in industry.

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