One-Pot Transformation of Ketoximes into Optically Active Alcohols and Amines by Sequential Action of Laccases and Ketoreductases or ω-Transaminases

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Abstract: An enzymatic one-pot process for asymmetric transformation of prochiral ketoximes into alcohols or amines was developed by sequential coupling of a laccase-catalysed deoximation either with a ketone reduction (ketoreductase, KRED) or bioamination (ω-transaminase, ω-TA) in aqueous medium. An accurate selection of biocatalysts provided the corresponding products in excellent enantiomeric excesses and overall conversions ranging from 83 to >99% for alcohols and 70 to >99% for amines. Likewise, the employment of exclusively 1% (w/w) of Cremophor®, a polyethoxylated castor oil, as co-solvent enabled to reach concentrations up to 100 mM in the chiral alcohols cascade.

Introduction

The oxime function is ubiquitous in nature and can be found in the structure of innumerable natural and synthetic bioactive compounds as well as in several metabolic pathways, being modified by the action of enzymes.[1] So for example, from bacterial origin the group of 2,2´-bipyridyl-containing natural products includes members bearing oximes such as pyrisulfoxins,[2] caerulomycins[3] and collismycins[4] which display antibacterial, antifungal and cytotoxic activities, and fungi like basidiomycete *Boreostereum vibrans* produce vibralactoximes with significant cytotoxic activity.[5] On the other hand, oximes are valuable synthetic precursors of ketones and aldehydes since those can be prepared from non-carbonyl precursors. Likewise, they also provide a facile entry to a wide variety of functionalities such as amines (reduction with metals or hydrides) and carboxamides (Beckmann rearrangement) which can be subsequently hydrolysed into acids, or nitriles (metal- or enzyme-catalysed dehydration).[6] However, from the vast number of synthetic transformations involving oximes, only very few are asymmetric processes. Particularly, the enantioselective reduction of *O*-substituted oximes, namely the boron-mediated reduction of oxime ethers and the rhodium-mediated reduction of oxime ethers led to amines or *N*-acylamines respectively, with good yields and high optical purity (Scheme 1, route a).[7] Similarly, a chemoenzymatic approach combining lipase/palladium catalysis provided optically active amines with very high enantiomeric excess although in their acetylated form (route b).[8]

In recent years considerable progress has been made in developing multistep one-pot processes since this strategy is expected to increase sustainability of chemical manufacture (minimization of waste production, time- and energy-consuming and purification steps).[9] This is particularly appealing when several biocatalytic reactions are integrated into multi-catalytic networks emulating Nature, as living organisms use a great number of enzymatic cascades (in different metabolic pathways) employing the same reaction media. Thus, biocatalysts are intrinsically "green" and aqueous media their natural environment.[10] Recently, we reported the first biocatalytic deoximation, which enabled to recover ketones from ketoximes by means of a laccase/TEMPO system.[11] Remarkably, the reaction took place in aqueous medium under mild reaction conditions, and the products were isolated in excellent yield without the need for chromatographic purification. Building on our interest in multistep enzymatic processes, herein we present a genuine enzymatic cascade to convert prochiral ketoximes into optically active alcohols or amines by the one-pot combination of the above biodeoximation with a subsequent stereoselective bioreduction (mediated by KREDs) or transamination reaction (mediated by ω-TAs) (route c). In fact, laccases have evolved from mere anti-pollutant reagents to smart biocatalysts for biooxidation processes,[12] including examples of catalytic networks in combination with enzymes such as lipases, oxidoreductases, ω-transaminases or enoate reductases,[13] although none involving oxime transformations.

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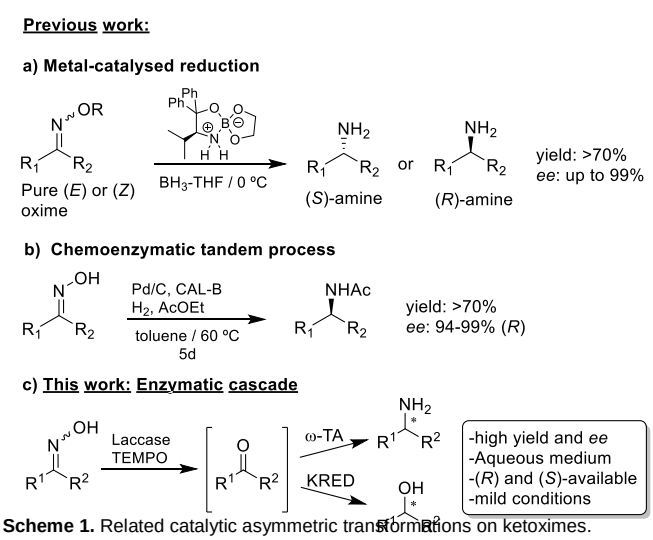


Scheme 1. Related catalytic asymmetric transformations on ketoximes.

Despite the merit of the previous synthetic approaches to convert oximes into optically active amines (Scheme 1), some shortcomings remain: *route a* (metal-catalysed reaction) demanded single-isomeric oxime ethers as starting materials since (*Z*)-oximes led to (*R*)-amines and (*E*)-ketoximes produce the (*S*)-counterparts, while for *route b* (chemoenzymatic route) the process suffered from long reaction times (5 days) and enabled exclusively (*R*)-amines as acetamides, which require very harsh conditions to be hydrolysed. Conversely, the novel enzymatic cascade would give facile entry to both enantiomers of chiral amines from *Z*/*E* mixtures of oximes under mild reaction conditions and in aqueous medium.

Results and Discussion

Nature reveals in the living cells how a perfect one-pot system works. Thus, metabolism is a perfect machinery of enzymatic networks where multistep reactions are mediated by enzymes playing in concert in an aqueous environment. Nevertheless, when mimicking these systems, a number of concerns must be circumvented to achieve the efficient implementation of such artificial enzymatic cascades.[14] A first challenge would be making the target biocatalysts compatible with respect to preferred pH, T, concentration and co-solvents profiles, but also specific activities and stability should be balanced and inhibition avoided. Particularly, the process envisaged in Scheme 1 (route c) seems more suitable for a sequential configuration than for a concurrent one seeing as: *i*) optimal pH for laccases and KREDs/ω-TAs are quite distant; more importantly, *ii*) if both catalysts are coexisting and functional simultaneously, the resulting chiral alcohol or amine could be oxidised by the laccase/TEMPO pair. Accordingly, it was planned initially a sequential one-pot two-steps process in which once the oxime cleavage is completed at pH 5.0 by the laccase, the pH would set to 7.0 and the KRED/ω-TA added to the medium. Theoretically, the laccase would be deactivated upon these conditions and the target chiral product prevented from further oxidation. Actually, it is a known fact that the laccases are highly influenced by pH, inactivated at basic pH because of binding of hydroxide anion to the copper ion at the active site and disrupting the internal electron transfer pathway.[12a]

In order to explore the viability of the coupled process, we set out to investigate as a bench reaction the biodeoximation of propiophenone oxime (**1a**) to propiophenone (**1b**) using the reported laccase/TEMPO system. Thus, the released **1b** has proven to be an excellent substrate for both KREDs and ω-TAs. With the knowledge that *Trametes versicolor* and TEMPO turned out to be the most effective pair of laccase and mediator,[11] we sought to parametrise the biotransformation in terms of temperature, concentration, and co-solvents with known compatibility with KREDs and ω-TAs. Thus, and upon the reported reaction conditions, the biodeoximation of **1a** was completed in 12 h in a citrate buffer 50 mM (pH 5.0) containing 10% v/v of acetonitrile (CH_3CN, Table 1, entry 1) at 20 ºC. First, a temperature of 40 ºC resulted in an acceleration of the process, taking 2 h for the consumption of 90% of **1a** (95% conversion after 12 h, entry 2). However, an increase to 50 ºC had a detrimental effect and enabled a conversion of 70% after 12 h, not evolving further due to enzyme deactivation (entry 3). Next, four co-solvents, such as dimethyl sulfoxide (DMSO), 1,4-dioxane, toluene and *tert*-amyl alcohol were screened under identical conditions at 10% v/v. As a result, very high conversions were reached in all the cases after 12 h (>95%, entries 4-7). Alternatively, the focus was placed on improving the ecological footprint of the process. Actually, although the greenness of biocatalysis is typically assumed, most biotransformations suffer from issues such as water consumption, wastewater production or unfavorable metrics, keeping them to reach real industrial impact.[15] Particularly, the poor solubility of most reagents in water leads to low substrate loadings and, by extension, poor economic performances. Likewise, the toxicity of the salts contained in some aqueous buffers is usually overlooked. Based on these considerations, the biodeoximation of **1a** was explored at 50 mM in a simplified medium consisting of water and 1% (w/w) of Cremophor®.[16] Thus, the reaction proceeded smoothly in such a low percentage of solubiliser and free of organic co-solvents (entry 8). Finally, the biotransformation was evaluated at 100 mM and 200 mM using 10% v/v of CH_3CN and 1% v/v of Cremophor®. In the first case, reactions rendered moderate conversion in the presence of CH_3CN (70% and 30% respectively, entries 9-10) meanwhile a high enzymatic performance was achieved owing to the employment of Cremophor® (>85%, entries 11-12).

Table 1. Parametrisation of the laccase-catalysed biodeoximation of propiophenone oxime (**1a**) in the presence of TEMPO and aerial O_2.[a]

Entry	Co-solvent	T (ºC)	[**1a**] (mM)	conv. (%)[b]
1	CH_3CN	20	50	>99
2	CH_3CN	40	50	95
3	CH_3CN	50	50	70
4	DMSO	20	50	95
5	1,4-Dioxane	20	50	97
6	Toluene	20	50	95
7	*t*-Amyl alcohol	20	50	97
8	Cremophor[c]	20	50	97
9	CH_3CN	20	100	70
10	CH_3CN	20	200	30
11	Cremophor[c]	20	100	90
12	Cremophor[c]	20	200	85

[a] Reaction conditions for entries 1-8: **1a** (50 mM), *T. versicolor* laccase (30 U), TEMPO (10% mol), citrate buffer 50 mM pH 5.0 (900 μL) and co-solvent (90 μL), stirring in an open vessel during 12 h. Experiments were performed in triplicate. [b] Determined by HPLC. [c] Used exclusively in 1% v/v.

Next, the substrate scope of the biotransformation was investigated. Thus, a series of ketoximes was selected (Figure 1), including those derived from propiophenone (**1a**-**4a**), acetophenone (**5a**-**12a**), non-benzylic (**13a**-**15a**), aliphatic (**16a**) and cyclic ketones (**17a**). Following the reaction conditions depicted in Table 1 (entry 1), ketoximes were in most cases quantitatively converted into the corresponding ketones after 20 h. Likewise, the products were isolated in high yields (>90%) by simple extraction without need for further purification. Conversely, **17a** led to very low conversion meanwhile the reaction did not work with **16a**, the only aliphatic substrate.

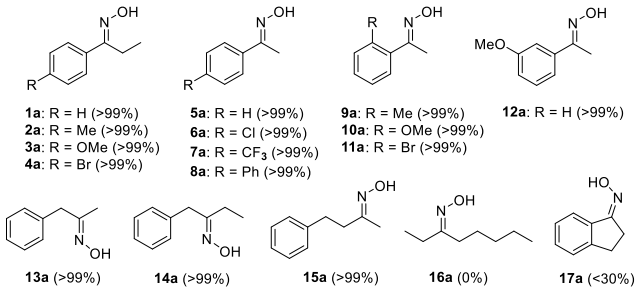


Figure 1. Enzymatic deoximation of ketoximes (conversion values in parenthesis).

Once assesed the viability of the biodeoximation with a variety of ketoximes, we focused on the first of the devised enzymatic cascades, namely that aimed at chiral alcohols. Thus, the initial deoximation of **1a** (50 mM) was performed in citrate buffer 50 mM pH 5.0 (containing 10% CH_3CN) and 20 ºC by the combined action of the laccase and TEMPO, the substrate being totally converted into ketone **1b** after 12 h. Then, the reaction mixture was diluted with a phosphate buffer 125 mM pH 7.0 (1 mM $NADP^+$) for a final substrate concentration of 25 mM, and the ketoreductase P1-A04 and *i*-PrOH for cofactor recycling and equilibrium shift, were subsequently added. Pleasantly, HPLC-monitoring showed quantitative reduction of the transiently formed ketone in 8 h, the resulting chiral alcohol (*R*)-**1c** displaying >99% *ee* (Table 1, entry 1). Seeing as the feasibility of the catalytic system, it looks clear that both laccase and TEMPO do not inhibit the KRED, which preserves the activity in terms of reaction rate and selectivity. Particularly, the complete conversion discarded a hypothetical oxidation of the target **1c** by the laccase, which was inactivated by the basic conditions of the second step. Next, and on the basis of the established reaction conditions, some ketoximes depicted in Figure 1 were subjected to this enzimatic one-pot two-steps sequence. Moreover, a careful selection of the KRED attending to previous reports ensured a high enantioselectivity towards the released intermediate ketones.[17] Interestingly, the study included purified enzymes from a commercial kit (Codexis)[18] and also two KREDs overexpressed in *E. coli* with opposite stereoselectivity, namely the (*R*)-selective alcohol dehydrogenase from *Lactobacillus kefir*[19] and the (*S*)-selective from *Rhodococcus ruber*.[20] As deduced from Table 2, the synthetic cascade worked efficiently with both purified/overexpressed enzymes and enabled to obtain the antipodes of chiral alcohols in very high *ee* and overall conversion. Particularly, a detrimental effect of the laccase/TEMPO system on the overexpressed KREDs was observed (see details in the ESI). The only required setting to solve this drawback was removing the insolubles (e.g., precipitated protein) by centrifugation after the deoximation step and adding to the supernatant the reagents for the further reduction. Under these experimental conditions, most of the selected alcohols were produced with very high conversions (entries 2,3,10,11,14-19). A further goal of the project was to enhance the substrate concentration for the cascade process, which could meet the parameters of a manufacture setting. Accordingly, the unique properties of Cremophor® (1% v/v) enabled a conversion of 85->99% for some selected deoximations run at 200 mM. Further dilution to 100 mM with the reaction medium required for the bioreduction provided, alternatively, the (*R*)-or (*S*)-enantiomer of alcohols **1c** and **4c** in 65-97% overall conversion and >99% *ee* (entries 4,5,12 and 13). Following an analogous procedure, the stepwise combination of the laccase-catalysed deoximation of ketoximes with a further bioamination would produce optically active amines. From the biocatalytic toolbox there are different classes of enzymes able to convert prochiral carbonyl compounds into amines: i) ω-TAs transfer a primary amino group from a donor substrate to an acceptor compound mediated by pyridoxal-5-phosphate (PLP);[21] ii) amino dehydrogenases (AmDHs) catalyze the NAD(P)H-dependent reductive amination of carbonyl compounds with ammonia;[22] iii) IREDs[23] and its recently discovered subclass

reductive aminases (RedAms)[24] accept secondary linear and cyclic amino donors and give entry to secondary and tertiary amines. Among, these, we focused on the first group and unlike KREDs, preliminar feasibility tests unveiled the inactivation of ω-TAs by Cremophor®. Accordingly, the deoximation for this enzymatic cascade should be set up in the presence of acetonitrile as co-solvent. Likewise, ω-TAs generally require lower substrate concentration than KREDs for an optimal enzymatic performance. With this premises, **1a** (50 mM) was subjected to deoximation by means of the treatment with the laccase/TEMPO system in citrate buffer 50 mM pH 5.0 (containing 10% CH_3CN) at 20 ºC following the previous procedure (Table 1, entry 1). After 12 h, the reaction mixture was submitted to pH adjustment with a phosphate buffer 100 mM pH 8.5 for a final substrate concentration of 17 mM and pH 7.5. Likewise, the resulting reaction medium contained *i*-$PrNH_2$ (1 M) and PLP (1 mM). Then, ω-transaminase ATA-025 and DMSO (2.5% v/v) were added, and after 24 h of shaking at 50 ºC the amine (*R*)-**1d** was obtained in enantiopure form and 85% of overall conversion (Table 3, entry 1). Identically, the employment of the stereocomplementary biocatalyst ATA-026 provided the enantiomer (*S*)-**1d** with high conversion (*c* = 88%) and >99% *ee* (entry 2). Likewise, the absence of by-products from an over-oxidation of the amine suggested the laccase-inactivation in the basic conditions of the second step. Encouraged by the success of this enzymatic catalytic system, we extended the methodology to some ketoximes from Figure 1. For this, the ω-TA was chosen for each particular ketone according to previous reports,[9c,25] from both a commercial kit (Codexis)[26] and three ω-TAs overexpressed in *E. coli*: the *S*-selective ω-TAs from *Chromobacterium violaceum* (Cv)[27] and (*S*)-*Arthrobacter* (ArS)[28] and the *R*-selective TAs from (*R*)-*Arthrobacter* (ArR)[29] (entries 3-16). Gratefully, the overall conversion was very high in most cases (*c* = from 70 to >99%), without traces of the starting ketoxime. With regards to the selectivity, the ω-TAs displayed perfect asymmetry towards most of the intermediate ketones (>99% *ee*), with identical values to those reported in the single bioamination. Again, the laccase/TEMPO system exerted some kind of inhibition on the overexpressed enzymes, which could be bypassed by avoiding the insolubles (see the ESI) and adding to the supernatant the reagents for the enzymatic amination. Under these experimental conditions all the selected amines were produced in quantitative conversion (entries 13-16).

The effective validation of both synthetic sequences highlights the suitability of multi-step enzymatic approaches run in one-pot fashion as a valuable alternative to existing methods. Importantly, the bioconversion of ketoximes into chiral amines does not require isomerically pure oximes and provides free amines as products. On the other hand, the enzymatic cascade aimed at chiral alcohols underlines the advantages of Cremophor® in the fields of chemocatalysis and biocatalysis and will open up new perspectives for further exploration of one-pot processes in aqueous media. Finally, the synthetic utility of this catalytic platform was showcased by performing a selected example, namely the conversion of ketoxime **4a** into alcohol **4c** on preparative scale (100 mg). As detailed in Table 2 (entry 13), **4a** underwent effective deoximation at 200 mM by the assistance of 1% (w/w) of Cremophor®. Once the oxime was completely consumed (HPLC analysis), the reaction mixture was diluted to 100 mM with the aqueous buffer for the bioreduction (containing NAD^+) and fed with the ADH from *R. ruber* and *i*-PrOH. Thus, the bioreduction of the formed ketone intermediate **4b** took place smoothly, (*S*)-**4c** being obtained with very high conversion (>95% overall conversion). Further filtration on silica gel rendered pure (*S*)-**4c** in 85% yield and >99% *ee.*

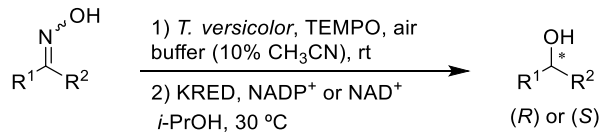
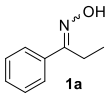
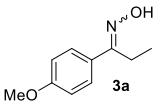
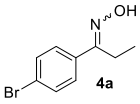
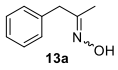
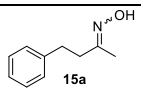


Table 2. One-pot sequential enzymatic transformation of ketoximes into optically active alcohols[a]

Entry	Substrate	Substrate conc. (mM)	KRED	Co-solvent (% v/v)	Product distribution (%)[b],[c]			*Ee* alcohol (%)[d]
					Ketoxime (a)	Ketone (b)	Alcohol (c)	
1	 **1a**	50	P1-A04	CH_3CN (10%)	<1	<1	>99	>99 (*R*)
2		50	*L. kefir*	CH_3CN (10%)	<1	<1	>99	>99 (*R*)
3		50	*R. ruber*	CH_3CN (10%)	<1	<1	>99	>99 (*S*)
4		200	*L. kefir*	Cremophor® (1%)	15	20	65	>99 (*R*)
5		200	*R. ruber*	Cremophor® (1%)	15	5	80	>99 (*S*)
6	 **3a**	50	P1-A04	CH_3CN (10%)	<1	15	85	>99 (*R*)
7		50	P3-H12	CH_3CN (10%)	<1	17	83	>99 (*S*)
8		200	*L. kefir*	Cremophor® (1%)	<1	94	6	-
9		200	*R. ruber*	Cremophor® (1%)	<1	50	45	>99 (*S*)
10	 **4a**	50	*L. kefir*	CH_3CN (10%)	<1	<1	>99	98 (*R*)
11		50	*R. ruber*	CH_3CN (10%)	<1	<1	>99	>99 (*S*)
12		200	P1-A04	Cremophor® (1%)	<1	4	96	>99 (*R*)
13		200	*R. ruber*	Cremophor® (1%)	<1	3	97	>99 (*S*)
14	 **9a**	50	*L. kefir*	CH_3CN (10%)	<1	85	15	-
15		50	*R. ruber*	CH_3CN (10%)	<1	<1	>99	>99 (*S*)
16	 **13a**	50	*L. kefir*	CH_3CN (10%)	<1	5	95	>99 (*R*)
17		50	*R. ruber*	CH_3CN (10%)	<1	5	95	>99 (*S*)
17	 **15a**	50	*L. kefir*	CH_3CN (10%)	<1	6	94	>99 (*R*)
19		50	*R. ruber*	CH_3CN (10%)	<1	9	92	>99 (*S*)

[a] Reaction conditions detailed in the ESI. The substrate concentration was diluted in half after the first step. Experiments were performed in triplicate. [b] Consumption of the ketoxime in the first step was checked by HPLC analysis. [c] The ratio of ketoxime (**a**), ketone (**b**), and alcohol (**c**) was measured by HPLC analysis. [d] Enantiomeric excess determined by chiral HPLC.

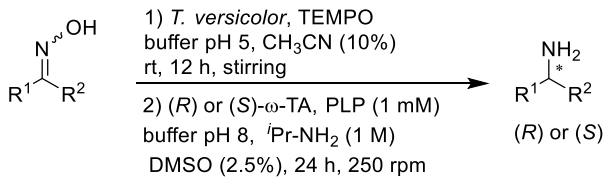
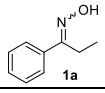
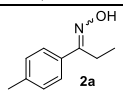
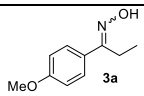
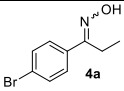
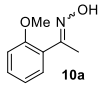
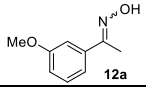
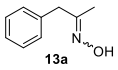
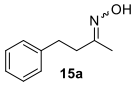


Table 3. One-pot sequential enzymatic transformation of ketoximes into optically active amines[a]

Entry	Substrate[b]	ω-TA	T (ºC)	Product distribution (%)[c],[d]			*Ee* amine (%)[e]
				Ketoxime (**a**)	Ketone (**b**)	Amine (**d**)	
1	 **1a**	ATA-025	50	<1	15	85	>99 (*R*)
2		ATA-026	50	<1	12	88	>99 (*S*)
3	 **2a**	ATA-025	50	<1	30	70	>99 (*R*)
4		ATA-251	50	<1	20	80	>99 (*S*)
6	 **3a**	ATA-024	50	<1	15	85	>99 (*R*)
7		ATA-251	50	<1	25	75	>99 (*S*)
8	 **4a**	ATA-024	50	<1	9	91	>99 (*R*)
9		ATA-237	50	<1	11	89	>99 (*S*)
10	 **10a**	ATA-033	50	<1	<1	>99	96 (*R*)
11		ATA-256	50	<1	8	92	>99 (*S*)
12	 **12a**	ATA-254	50	<1	<1	>99	>99 (*S*)
13	 **13a**	ArR	30	<1	<1	>99	>99 (*R*)
14		Cv	30	<1	<1	>99	>99 (*S*)
15	 **15a**	ArR	30	<1	<1	>99	>99 (*R*)
16		ArS	30	<1	<1	>99	>99 (*S*)

[a] Reaction conditions detailed in the ESI. Experiments were performed in triplicate. [b] Substrate concentration was fixed at 50 mM for the biodeoximation and 17 mM for the subsequent bioamination. [c] Consumption of the ketoxime in the first step was checked by HPLC analysis. [c] The ratio of ketoxime (**a**), ketone (**b**), and amine (**d**) was measured by HPLC analysis. [d] Enantiomeric excess determined by chiral HPLC.

Conclusions

We disclose an expedient, stereoselective and operationally simple protocol for the synthesis of either optically active amines or alcohols from ketoximes, through the sequential one-pot combination of: i) an enzymatic deoximation mediated by a laccase/TEMPO catalytic system; ii) subsequent enantioselective bioamination or bioreduction of the transiently formed ketones. In both cases, the desired final products were obtained in high yields and excellent enantiomeric excesses. Likewise, it is worth noting the use of a one-pot methodology, in which the aqueous reaction medium from the deoximation step feeds the enzymatic amination/reduction.

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Keywords: one-pot reaction • oxime • alcohol dehydrogenase • transaminase • enzymatic cascade

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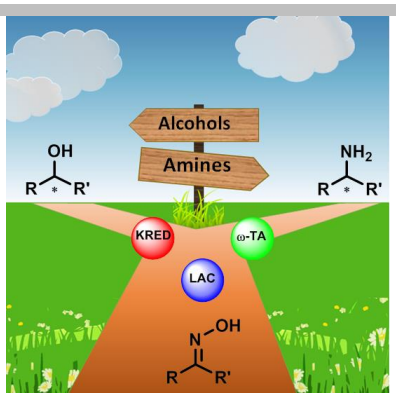

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Page No. – Page No.

One-Pot Transformation of Ketoximes into Optically Active Alcohols and Amines by Sequential Action of Laccases and Ketoreductases or ω-Transaminases