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Chemoenzymatic deracemization of lisofylline catalyzed by a (laccase/TEMPO)-alcohol dehydrogenase system†

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Lisofylline (LSF) is a synthetic methylxanthine active agent exhibiting potent anti-inflammatory and immunomodulatory properties; therefore, it has been widely investigated as a promising drug candidate for treating various autoimmune disorders, including type 1 diabetes. In this study, we report on developing a sequential chemoenzymatic one-pot two-step deracemization protocol for racemic LSF. This task was accomplished in a stereo-complementary manner *via* a tandem bi-enzymatic oxidation-reduction reaction sequence composed of (i) non-selective chemoenzymatic aerobic oxidation of LSF to pentoxifylline (PTX) catalyzed by commercially available laccase from *Trametes versicolor* (LTV) and 2,2,6,6-tetramethylpiperidinyloxy radical (TEMPO) as a redox mediator, and (ii) stereoselective bioreduction of *in situ* formed PTX to give enantiomeric LSF, which was catalyzed by home-made lyophilized *E. coli* cells harboring overexpressed alcohol dehydrogenases (ADHs) with complementary stereospecificity. Firstly, a multi-step optimization procedure of LTV/TEMPO-catalyzed oxidation of LSF allowed achieving dramatic improvement of the conversion rates from an initial 16% up to 95%, demonstrating the high synthetic potency of this method compared to traditional chemical reactions requiring toxic oxidants used in stoichiometric amounts. In turn, separate stereoselective bioreductions of PTX using recombinant ADHs from *Rhodococcus ruber* (*E. coli*/ADH-A) and *Lactobacillus kefir* (*E. coli*/LK-ADH Prince) furnished both LSF enantiomers (>99% ee) with high 93–94% conversion and in 65–67% yield range, respectively. The coupling of the above-mentioned chemoenzymatic steps afforded both antipodes of LSF on a preparative scale (0.16 mmol of racemic LSF) in the range of 56–67% yield and 94% ee depending on the employed ADHs.

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1. Introduction

Lisofylline (LSF) is a powerful pharmaceutical molecule whose strong anti-inflammatory and immunomodulatory properties¹ are useful in the effective prevention and treatment of various autoimmune disorders, such as inflammatory bowel disease (*i.e.*, Crohn's disease),² insulin-dependent type 1 diabetes,³ bronchial asthma,⁴ *etc.*, as well as in attenuating the side effects (*i.e.*, neutropenic infections) of chemotherapy of cancers.⁵ In the case of LSF, pharmacokinetic and pharmacodynamic studies revealed that the desired biological activities reside only in the (*R*)-enantiomer, which is, by the

way, several hundred-fold more potent at inhibiting the activity of inflammatory cytokines than its parent molecule, pentoxifylline (PTX).⁶ Due to the essential pharmacological significance of (*R*)-LSF, the elaboration of asymmetric methods of its synthesis has attracted significant attention from synthetic laboratories. Among biocatalytic attempts worth mentioning are (*R*)-stereoselective bioreduction of PTX catalyzed by microbial whole cells of *Saccharomyces cerevisiae* (wine yeast)⁷ or resting cells of *Lactobacillus kefir* DSM 20587⁸ as well as purified (*R*)-specific alcohol dehydrogenase (ADH) isolated from *Lactobacillus kefir* (LK-ADH)⁹ (Fig. 1).

Alternative enzymatic approaches relying on the microbial bioreduction of PTX using cell cultures of *Rhodotorula rubra* DSM 5436 exhibiting Prelog stereoselectivity¹⁰ or chemo- and stereoselective hydroxylation of 1-*N*-hexyl-theobromine catalyzed by wild-type¹¹ or engineered human cytochrome P450 3A4 monooxygenases¹² seem to be less attractive from the viewpoint of undesired absolute configuration or low enantiomeric excess values of the obtained non-racemic LSF, respectively. With the aim to expand the synthetic toolbox for the synthesis of the titled API, we have recently developed

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Fig. 1 Overview of different biocatalytic strategies for the synthesis of enantiomeric lisofylline (LSF).

two independent variants of lipase-catalyzed kinetic resolution (KR) of racemic LSF by either using a classic transesterification approach with vinyl acetate as acyl donor¹³ or utilizing α -angelica lactone as a nonconventional irreversible acylating agent for chromatography-free KR.¹⁴ However, enzymatic KR of *sec*-alcohols suffers from low reaction yields, which renders the use of this approach unsustainable for industrial applications.

Biocatalytic cascades represent an attractive strategy in the synthesis of optically active compounds.¹⁵ This assumption stems from the fact that one-pot combinations of sequential catalytic reactions improve step economy, lower production costs, and generate less waste to be recycled. In this context, elaboration of novel synthetic methodologies that would

efficiently couple sequential non-selective oxidation of racemic alcohols with biocatalytic stereoselective reductions of the *in situ* generated ketones through one-pot cascades under mild conditions has received considerable attention.¹⁶ Initially, deracemizations of *sec*-alcohols *via* a biocatalytic oxidation-reduction sequence have been achieved mainly using whole microbial cells,¹⁷ enantiocomplementary ADHs¹⁸ or single ADH-catalyzed biohydrogen transfer mediated by α -chloro ketones.¹⁹ Furthermore, a combination of organocatalytic oxidation of racemic alcohols catalyzed by Ir-complex,²⁰ sodium hypochlorite (NaOCl) and 2-azaadamantane *N*-oxyl (AZADO),²¹ Zr-beta zeolite²² or iodine/(2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)²³ system with ADH-catalyzed asymmetric bioreduction of the formed ketones have appeared as interesting alternatives



to obtain optically pure hydroxyl compounds. In the last few years, a relatively novel catalytic system composed of laccase from *Trametes versicolor* (LTv), stable nitroxyl radical TEMPO, and the respective ADHs has been successfully applied in sustainable deracemization of various benzylic,²⁴ allylic,²⁵ and propargylic²⁶ alcohols using a linear one-pot bio-redox cascade strategy. This methodology was developed on the basis of a pioneering paper published by Fabbrini *et al.*,²⁷ which was the first that reported on chemoenzymatic oxidation of alcohols with oxygen catalyzed by laccase from *Trametes versicolor* and mediated by TEMPO. However, the LTv/TEMPO-ADH system, despite being proven useful for deracemization of a broad spectrum of activated *sec*-alcohols, is still underexploited toward unactivated substrates, such as aliphatic, alicyclic and/or heteroaromatic hydroxy derivatives. Therefore, extending the substrate scope for more demanding compounds in terms of oxidation of their secondary hydroxyl groups remains a challenging task for (bio)organic chemists.

In continuation of our interest in the syntheses of enantiomerically pure APIs *via* biocatalytic methodologies,²⁸ we report here an unprecedented synthetic strategy toward both enantiomers of lisofylline (LSF) achieved by the employment of tandem catalysis based on one-pot reversed redox transformations mediated by the LTv/TEMPO-ADH system in an aqueous medium

supplemented with organic co-solvents and under mild conditions.

2. Results and discussion

In chemoenzymatic one-pot redox-driven deracemization of secondary alcohols using laccase/TEMPO and ADH as the conjugated catalysts, the non-selective oxidation of the respective racemate seems to be a more demanding process from the synthetic point of view (Fig. 2).

It is especially true when one desires to obtain optically pure compounds with this methodology; then, an oxidative step must proceed in >99% yield to provide quantitative amounts of prochiral ketone intermediate for the subsequent ADH-catalyzed bioreduction. Otherwise, each 1% less conv. prompts a 1% drop in an enantiomeric excess of the isolated non-racemic product even if the ADH-catalyzed reaction step remains fully stereoselective.

We commenced our study by optimizing the LTv/TEMPO-catalyzed non-selective oxidation of lisofylline (*rac*-1) using air/oxygen as an oxidant. In the first step, we examined the addition of various water-miscible and water-immiscible organic solvents, within which the employed substrate could form a homogenous solution, and gave rise to monophasic (in the case of DMSO, 1,4-dioxane, MeOH, EtOH, CH₃CN,



Fig. 2 (A) Schematic representation of LTv/TEMPO-ADH-mediated linear deracemization of *sec*-alcohols. (B) A plausible mechanism of laccase/TEMPO-mediated non-selective oxidation of *sec*-alcohols using air/O₂ as the oxidant followed by stereoselective bioreduction of the ketone intermediates.



acetone, THF, 2-PrOH) and/or biphasic (in the case of EtOAc, CH₂Cl₂, *tert*-amyl alcohol, PhCH₃) reaction systems with an oxygen-saturated citrate buffer (Table 1), respectively. The total volume of the reaction medium was set at a 1.6 mL level, while the minimum amount of the supplemented organic co-solvent (10–50% (v/v) in ratio to citrate buffer) relied strictly on the solubility of *rac*-1. Moreover, to minimize limitations concerning mass transfer issues from one side and to avoid the splitting of the reactant and catalysts around the vial walls from the other side, a gentle magnetic stirring (150 rpm) was applied in all cases. The solvent optimization showed that oxidation of *rac*-1 is favored in acetone, EtOAc, THF, CH₂Cl₂, *tert*-amyl alcohol, and PhCH₃, which indicates that no significant effects from the polarity of the solvents are expected to play a decisive role in this process. The highest conversion for *rac*-1 was observed in acetone and EtOAc. In contrast, the most detrimental effect on the activity of the laccase/TEMPO system exhibited low-molecular alcohols (MeOH, EtOH, and 2-PrOH) and highly polar DMSO. This observation can be either rationalized by strong promotion of protein unfolding, leading to denaturation of the enzyme in the presence of DMSO, or attributed to an inhibition mechanism caused by alcohols. Moreover, the short-chain alcohols used in a large molar excess can also compete with the substrate *rac*-1 to be oxidized by TEMPO-oxoammonium species. In turn, despite a high concentration of acetone (50% (v/v)), laccase from *Trametes versicolor* turned out to be significantly less vulnerable to inactivation effects when compared to the rest of the polar solvents (*i.e.*, DMSO, 1,4-dioxane, MeOH, EtOH,

and CH₃CN). This is quite an interesting result as in the case of many other studied fungal laccases, the complete inhibition of their catalytic activity was observed mainly at 50% organic co-solvent concentration.²⁹ Since the most efficient oxidation of *rac*-1 has been found in an aqueous citrate buffer/acetone mixture, this medium was chosen for further investigations. We arbitrarily omitted using EtOAc in later experiments as this solvent formed a biphasic system with an aqueous phase and is less volatile than acetone, which during the reaction progress is easily eliminated *via* evaporation.

Next, to improve the ecological footprint of the process, the influence of the amount of acetone (10–50% (v/v)) on the % conv. of *rac*-1 was examined (Table 2). In addition, the low oxidation rates of *rac*-1 observed in previous experiments forced us to extend the reaction time up to 96 h. It turned out that 20% (v/v) acetone was beneficial for the efficiency of the LTV/TEMPO-mediated oxidation of *rac*-1, leading to a 2.5-fold improvement of the % conversion. It is a somehow interesting result as lower than 50% (v/v) acetone concentrations resulted in the formation of suspensions due to the limited solubility of lisofylline (*rac*-1). In this case, the improvement in the reaction rates might be twofold: acetone deactivates the enzyme and/or the substrate *rac*-1 exhibits inhibitory potency toward laccase. In turn, lower than 20% (v/v) co-solvent concentration decreased the solubility of *rac*-1 to such an extent that the reaction progress ceased.

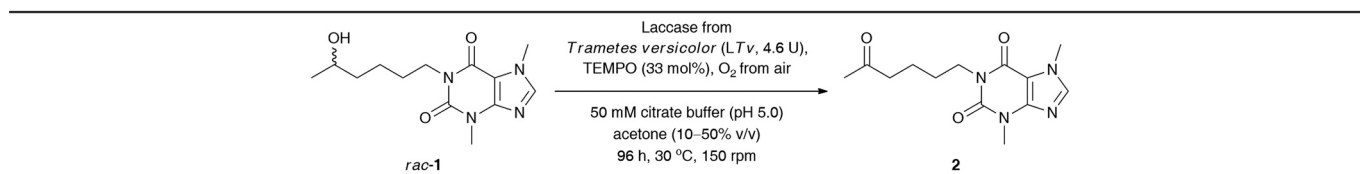
Since laccases (EC 1.10.3.2) are multi-copper-containing oxidases,³⁰ we found it crucial for this study to investigate

Table 1 Analytical-scale studies on the laccase-catalyzed bio-oxidation of lisofylline (*rac*-1, 50 mM) in the presence of TEMPO and aerial O₂ – screening of co-solvents

Entry	Co-solvent ^{a,b} (log <i>P</i>)	Co-solvent amount ^c (% v/v)	Conv. ^d (%)
1	DMSO (−1.49)	20	N.D. ^e
2	1,4-Dioxane (−0.31)	30	9
3	MeOH (−0.27)	30	N.D. ^e
4	EtOH (0.07)	50	N.D. ^e
5	CH ₃ CN (0.17)	50	5
6	Acetone (0.20)	50	16
7	EtOAc (0.29)	20	16
8	2-PrOH (0.38)	50	N.D. ^e
9	THF (0.40)	20	12
10	CH ₂ Cl ₂ (1.01)	15	13
11	<i>tert</i> -Amyl alcohol (1.09)	20	13
12	PhCH ₃ (2.52)	50	10

^a Reaction conditions: *rac*-1 (23 mg, 0.08 mmol, 50 mM final conc.), *T. versicolor* laccase (LTV, 7 mg, 4.6 U), TEMPO (4.1 mg, 33% mol), oxygenated citrate buffer (50 mM, pH 5.0), co-solvent (15–50% (v/v)) 1.6 mL final volume, 16 h, 30 °C, stirring in an open-to-air test vial (150 rpm, magnetic stirrer). ^b Logarithm of the partition coefficient of a given solvent between *n*-octanol and water according to ChemBioDraw Ultra 13.0 software indications. ^c Minimal amount of co-solvent in which a homogenous solution of the substrate *rac*-1 could be formed. ^d Conversion values (%) (*i.e.*, consumption of substrate *rac*-1) were determined by GC analyses after derivatization of the crude mixture with *N,O*-bis(trimethylsilyl)acetamide (BSA) as a silylating reagent. ^e Not detected.



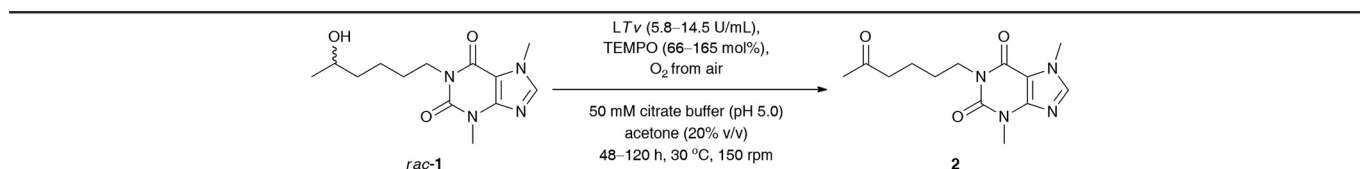
Table 2 Analytical-scale studies on the laccase-catalyzed bio-oxidation of isofylline (*rac-1*, 50 mM) in the presence of TEMPO and aerial O₂ – effect of acetone amount

Entry	Amount of acetone ^a (% v/v)	Conv. ^b (%)
1	10	30
2	20	40
3	30	38
4	40	26
5	50	28

^a Reaction conditions: *rac-1* (23 mg, 0.08 mmol, 50 mM final conc.), *T. versicolor* laccase (LTv, 7 mg, 4.6 U), TEMPO (4.1 mg, 33% mol), oxygenated citrate buffer (50 mM, pH 5.0), acetone (10–50% (v/v)) 1.6 mL final volume, 96 h, 30 °C, stirring in an open-to-air test vial (150 rpm, magnetic stirrer). ^b Conversion values (%) (*i.e.*, consumption of substrate *rac-1*) were determined by GC analyses after derivatization of crude mixture with *N,O*-bis(trimethylsilyl)acetamide (BSA) as a silylating reagent.

the effect of the presence of Cu²⁺ ions on the catalytic activity of *T. versicolor* laccase and indirectly on the whole LTv/TEMPO system (for details, see the ESI[†]). Moreover, inspired by the reported stimulatory effect of Cu²⁺, Zn²⁺, Fe²⁺, and Mg²⁺ on the laccases' activity,³¹ we also decided to determine the influence of different double-positive metal ions on the % conv. of the LTv/TEMPO-catalyzed oxidation of *rac-1*. In this regard, the reaction mixtures performed in citrate buffer (50 mM, pH 5.0) and acetone (20% (v/v)) for 24 h at 30 °C were supplemented with 1 mM final conc. of MgSO₄, FeCl₂·4H₂O, FeSO₄·7H₂O, CoCl₂·6H₂O, ZnSO₄·7H₂O, or CuSO₄·5H₂O. The control reactions were assayed without added metal ions and in two variants with aerial oxygen as well as under an O₂ atmosphere incorporated through an O₂-filled balloon. The results indicated that in major cases, there was no positive influence of metal ions on the activity of the LTv/TEMPO system in the tested reactions. Since the previous optimizations were unsuccessful in finding significant

enhancements on bio-oxidation of *rac-1* and still a relatively high quantity of unreacted substrate remained in the reaction mixture, therefore, the reoxidation of the crude product for the subsequent 24 h through the addition of LTv and TEMPO was necessary to improve the conversion (Table 3, entry 1). Disappointingly, such a single treatment failed to give the expected results, so further studies were carried out toward a sequential increase of the amount of both laccase and oxyradical mediator. It is noteworthy that for this purpose, three separate reactions were carried out with sequential addition of laccase (7 mg) and TEMPO (4.1 mg) after every 24 h, without changing the initial volume of the medium (Table 3, entries 2–4). Analysis of the data presented in Table 3 revealed that in order to obtain a reasonably high conversion of *rac-1* (>95%), the oxidation process had to be extended to 120 h, and the overall amounts of laccase and TEMPO had to be increased to 14.5 U mL⁻¹ and 165 mol%, respectively.

Table 3 Analytical-scale studies on the laccase-catalyzed bio-oxidation of isofylline (*rac-1*, 50 mM) in the presence of TEMPO and aerial O₂ – effect of the reaction time and the laccase/TEMPO amount

Entry	Laccase ^a (U mL ⁻¹)	TEMPO (mol%)	<i>t</i> (h)	Conv. ^b (%)
1	5.8	66	48	55
2	8.7	99	72	82
3	11.6	132	96	87
4	14.5	165	120	95

^a Reaction conditions: *rac-1* (23 mg, 0.08 mmol, 50 mM final conc.), *T. versicolor* laccase (LTv, 7 mg, 4.6 U, *ca.* 2.9 U mL⁻¹) and TEMPO (4.1 mg, 33% mol) – each portion added sequentially after 24 h, TEMPO (4.1 mg, 33% mol) – each portion added sequentially after 24 h, oxygenated citrate buffer (50 mM, pH 5.0) and acetone (20% (v/v)) 1.6 mL final volume, 48–120 h, 30 °C, stirring in an open-to-air test vial (150 rpm, magnetic stirrer). ^b Conversion values (%) (*i.e.*, consumption of substrate *rac-1*) were determined by GC analyses after derivatization of the crude mixture with *N,O*-bis(trimethylsilyl)acetamide (BSA) as a silylating reagent.



Table 4 Preparative-scale laccase-catalyzed bio-oxidation of lisofylline (*rac-1*, 50 mM) in the presence of TEMPO and aerial O₂


Entry	LTv (U mL ⁻¹)/TEMPO ^a (mol%)	t (h)	Conv. ^b (%)	Yield ^c (%)
1	8.7/99	72	60	48
2	11.6/132	96	80	75
3	14.5/165	120	95	68
4	17.4/198	144	93	63
5	8.7/99 ^d	120	86	81
6	14.5/33	120	62	56

^a Reaction conditions: *rac-1* (46 mg, 0.16 mmol, 50 mM final conc.), *T. versicolor* laccase (LTv, 14 mg, 9.2 U, ca. 2.9 U mL⁻¹) and TEMPO (8.2 mg, 33% mol) – each portion added sequentially after 24 h, oxygenated citrate buffer (50 mM, pH 5.0), acetone (20% (v/v)) 3.2 mL final volume, 72–144 h, 30 °C, stirring in an open-to-air test vial (150 rpm, magnetic stirrer). ^b Conversion values (%) (*i.e.*, consumption of substrate *rac-1*) were determined by GC analyses after derivatization of the crude mixture with *N,O*-bis(trimethylsilyl)acetamide (BSA) as a silylating reagent. ^c Isolated yield after column chromatography packed with SiO₂. ^d *rac-1* (46 mg, 0.16 mmol, 50 mM final conc.), *T. versicolor* laccase (LTv, 14 mg, 9.2 U, ca. 2.9 U mL⁻¹) and TEMPO (8.2 mg, 33% mol) – each portion added sequentially after 48 h (*i.e.*, the second portion was added after 48 h, and the third portion was added after 96 h), oxygenated citrate buffer (50 mM, pH 5.0), acetone (20% (v/v)) 3.2 mL final volume, 120 h, 30 °C, stirring in an open-to-air test vial (150 rpm, magnetic stirrer).

Next, our efforts were devoted to evaluating the efficiency of the laccase-mediated oxidation step performed on a larger scale of the employed substrate *rac-1* (0.16 mmol) (Table 4). The best result in terms of the achieved % conv. was obtained in the reaction conducted for 120 h with *T. versicolor* laccase (14.5 U mL⁻¹) and 165 mol% TEMPO. However, higher isolated yield for the desired ketone **2** was detected when the oxidation of *rac-1* was performed with *T. versicolor* laccase (11.6 U mL⁻¹) and 132 mol% TEMPO, and stopped after 96 h. This can be attributed to lower contamination from redundant laccase and TEMPO co-catalysts, and thus fewer drawbacks were encountered during purification of the crude product *via* preparative column chromatography. Once again, further extension of the reaction time failed to achieve quantitative conversions of *rac-1*.

This phenomenon can be rationalized by the so-called suicide inactivation of laccases, which is observed at high substrate conversions when the reactive groups in the protein (or the associated glycosyl moieties located on the periphery of the enzyme) are oxidized by the accumulated oxoammonium cation.³² Therefore, we decided to extend our optimizations by investigating the effect of a lower concentration of TEMPO on the sequential oxidation of *rac-1* (Table 4, entries 5 and 6). In the first case, the amounts of TEMPO and laccase have been reduced by half with respect to previous conditions. In the second attempt, we used only a catalytic amount of the mediator (33 mol%) coupled with laccase sequentially added in 24 h intervals at the same level as before (14.5 U mL⁻¹ in total). Unfortunately, both trials revealed that it was not the TEMPO that was responsible for the deactivation of laccase. Therefore, the oxidation of *rac-1* required stoichiometric amounts of the mediator as well as increased amounts of laccase for the efficient regeneration of the oxoammonium species of TEMPO, which is crucial for the oxidation step.

In the next step, stereoselective reduction of prochiral pentoxifylline (**2**) catalyzed by whole-cell biocatalysts was investigated to obtain optically pure lisofylline (*non-rac-1*). This task was accomplished using either wild-type microbial strains or recombinant alcohol dehydrogenases overexpressed in *Escherichia coli* cells (*E. coli*/ADHs) (Table 5). All the employed wild-type microorganisms (Table 5, entries 1–8) have been prepared by using standard cultivation conditions and lyophilized.³³ In turn, the *E. coli*/ADH biocatalysts that originated from *Ralstonia* sp. (*E. coli*/RasADH³⁴), *Sphingobium yanoikuyae* (*E. coli*/SyADH³⁵), *Rhodococcus ruber* (*E. coli*/ADH-A³⁶), *Lactobacillus brevis* (*E. coli*/LB-ADH³⁷) and *Lactobacillus kefir* (*E. coli*/Lk-ADH-Lica,³⁸ *E. coli*/LkADH,³⁹ *E. coli*/Lk-ADH Prince⁴⁰) have been prepared as indicated in the appropriate literature. The catalytic behavior of the above-mentioned panel of wild-type microorganisms and *E. coli*/ADHs (10 mg of dried cells) was tested in bioreduction processes of **2** (10 mM final conc.) under standard conditions [*i.e.*, 100 mM Tris-HCl buffer (pH 7.5) in the presence of NADH (0.5 mM) with 2.5% (v/v) DMSO due to the low solubility of **2** in aqueous medium, without air access for 48 h at 30 °C and 250 rpm] together with an *in situ* NAD(P)H regeneration system. Due to economic demands and the fact that the “cellular machinery” possesses metabolic pathways responsible for the efficient regeneration of nicotinamide cofactors, we decided to use an excess of isopropanol (2-PrOH) as a sacrificial co-substrate. Moreover, all the recombinant ADHs employed herein have already been confirmed to catalyze ketone reduction and cofactor recycling simultaneously in the presence of high concentrations of 2-PrOH (even up to 80% (v/v) in the case of *E. coli*/ADH-A⁴¹) as a reducing agent *via* hydrogen transfer. Therefore, in all the studied reactions, 10% (v/v) 2-PrOH was used with respect to Tris-HCl buffer, which additionally enhanced the solubility of substrate **2** in the aqueous phase.



Table 5 Analytical-scale studies on stereoselective reduction of pentoxifylline (**2**, 10 mM) with different biocatalysts after 48 h


Entry	Biocatalyst ^a	Strain	Additive	Conv. ^b (%)	ee _p ^c (%) (Config. ^d)
1	<i>Komagataella phaffi</i> / <i>Pichia pastoris</i>	ATCC 76273	20 mM glucose	N.D. ^e	N.A. ^f
2	<i>Pseudomonas</i> sp.	DSM 6978	20 mM glucose	N.D. ^e	N.A. ^f
3	<i>Arthrobacter</i> sp.	DSM 7325	20 mM glucose	83	80 (R)
4	Isolate <i>Actinomyces</i> sp. SRB-AN040	FCC025	20 mM glucose	54	97 (S)
5	Isolate <i>Actinomyces</i> sp. SRB-AN053	FCC027	20 mM glucose	92	>99 (S)
6	Isolate <i>Actinomyces</i> sp. ARG-AN024	FCC014	20 mM glucose	93	>99 (S)
7	Isolate ARG-AN025	FCC015	20 mM glucose	94	99 (S)
8	Isolate USA-AN012	FCC021	20 mM glucose	92	99 (S)
9	<i>E. coli</i> /RasADH	—	20 mM glucose	95	97 (S)
10	—	—	—	95	97 (S)
11	<i>E. coli</i> /SyADH	—	20 mM glucose	87	>99 (S)
12	—	—	—	N.D. ^e	N.A. ^f
13	<i>E. coli</i> /ADH-A	—	20 mM glucose	98	99 (S)
14	—	—	—	98	>99 (S)
15	<i>E. coli</i> /LB-ADH	—	20 mM glucose	41	86 (R)
16	—	—	—	37	39 (R)
17	<i>E. coli</i> /Lk-ADH-Lica	—	20 mM glucose	98	53 (R)
18	—	—	—	97	60 (R)
19	<i>E. coli</i> /Lk-ADH	—	20 mM glucose	96	90 (R)
20	—	—	—	97	81 (R)
21	<i>E. coli</i> /Lk-ADH prince	—	20 mM glucose	98	96 (R)
22	—	—	—	98	>99 (R)

^a Reaction conditions: **2** (10 mM final conc.), lyophilized biocatalyst (10 mg), 20 mM glucose, 0.5 mM NADH, 0.1 M Tris-HCl buffer (pH 7.5)/2-ProOH (500 μ L, 90:10, v/v), DMSO (2.5% (v/v)), 48 h, 30 $^{\circ}$ C, 250 rpm (laboratory shaker). ^b Conversion values (%) (*i.e.*, consumption of substrate **2**) were determined by GC analyses after derivatization of the crude mixture with *N,O*-bis(trimethylsilyl)acetamide (BSA) as a silylating reagent. ^c Determined for *non-rac-1* by chiral HPLC analysis using a Chiralcel AD-H column. ^d Absolute configuration of optically active lisofylline (*non-rac-1*) was established by comparison of HPLC picks elution order with enantiomeric standards. The major enantiomer is shown in parentheses. ^e Not detected. ^f Not applicable because there was no detectable conversion.

In turn, supplemental glucose (20 mM final conc.) was chosen to act as a carbon source and alternative cofactor-recycling system for NAD(P)H *via* the Embden–Meyerhof–Parnas (EMP) metabolic pathway.

The results of this screening showed that among the tested wild-type strains, four out of eight are capable of (*S*)-stereoselectively reducing pentoxifylline (**2**), giving access to (*S*)-(+)-**1** in 92–94% conv. and enantiopure form (from 99% ee to >99% ee) (Table 5, entries 5–8). In contrast, only one strain from *Arthrobacter* sp. exhibited anti-Prelog selectivity; however, (*R*)-(-)-**1** was obtained in moderate 83% conv. and 80% ee. When analyzing the reactions catalyzed by *E. coli*/ADHs one could observe that the following enzyme preparations from *Ralstonia* sp. (*E. coli*/RasADH), *Sphingobium yanoikuyae* (*E. coli*/SyADH), and *Rhodococcus ruber* (*E. coli*/ADH-A) displayed Prelog selectivity, while in contrast, ADHs from *Lactobacillus brevis* (LB-ADH) and *Lactobacillus kefir* (Lk-ADHs) exhibited the reversed stereopreference toward **2**, leading to the anti-Prelog product (*R*)-(-)-**1**. The best results in terms of % conv. and % ee values were obtained in the reactions conducted without supplementation of glucose and catalyzed by both *E. coli*/ADH-A and *E. coli*/Lk-ADH Prince, which allowed isolation of

optically pure (*S*)-(+)-**1** and (*R*)-(-)-**1** with high 98% conversions. To our surprise, we found that *E. coli*/SyADH, *E. coli*/LB-ADH, and *E. coli*/Lk-ADH required supplementation of 20 mM glucose to retain the desired catalytic activity and/or selectivity. In the case of *E. coli*/LB-ADH and *E. coli*/Lk-ADH, from moderate to significant drops in enantiomeric excess of (*R*)-(-)-**1** (9–47 Δ % ee) were detected without a detrimental influence on the conversions (Table 5, entries 15 *vs.* 16 and 19 *vs.* 20), respectively. In sharp contrast, *E. coli*/SyADH turned inactive without glucose since even traces of bioreduction product were not observed (Table 5, entry 12). The effect of sugar additives has already been investigated for ADHs⁴² and is suggested to be responsible for compacting these enzymes into a protein globule, which streamlines the efficiency of the hydride transfer crucial for catalysis and decreases the activation energy. It is also worth underlining that although most of the tested *E. coli*/ADH preparations overexpress NADPH-dependent alcohol dehydrogenases (except *E. coli*/ADH-A), we decided to supplement the reaction medium with a catalytic amount of a cheaper and more stable NADH cofactor than its phosphorylated counterpart. Surprisingly, this manipulation did not deteriorate the outcome of the bioreductions of **2**.





Scheme 1 Stereo-complementary bioreductions of pentoxifylline (**2**) catalyzed by *E. coli*/ADH-A or *E. coli*/Lk-ADH Prince, affording enantiomerically pure lisofylline [(S)-(+)-**1** and (R)-(-)-**1**].

Based on preliminary screening, the feasibility of the ADH-catalyzed bioreductions of **2** was further demonstrated through up-scaling of these reactions to 0.16 mmol of the substrate (Scheme 1). Semi-preparative scale stereo-complementary bioreductions of **2** using (S)-selective *E. coli*/ADH-A or (R)-selective *E. coli*/Lk-ADH Prince furnished both LSF enantiomers, (S)-(+)-**1** and (R)-(-)-**1**, in 65–67% isolated yield at 93–94% conv., depending on the applied ADH.

Finally, the last task was to combine the afore-optimized mechanistically reversed redox transformations into stereo-complementary ‘one-pot two-step’ biocatalytic cascades. The synthetic value of the novel protocol, giving the possibility to access both optical antipodes of the title API, was demonstrated on a preparative scale (Scheme 2). The coupling of *T. versicolor* laccase (14.5 U mL⁻¹) and 165 mol% TEMPO [both added sequentially in portions every 24 h: LTV (14 mg, 9.2 U) and TEMPO (8.2 mg, 33 mol%)] with the respective ADH (15 mg mL⁻¹) and preservation of the appropriate reaction conditions for both the oxidation (pH 5.0, magnetic stirring, 150 rpm) and the bioreduction (pH 7.5, orbital shaking, 250 rpm) steps afforded (S)-(+)-**1** in 56% yield and 94% ee in the case of *E. coli*/ADH-A, and (R)-(-)-**1** in 31% yield and 92% ee in the case of *E. coli*/Lk-ADH Prince. The decreased reaction yield for LTV/TEMPO-*E. coli*/Lk-ADH Prince system might be due to a partial deactivation of the alcohol dehydrogenase in the presence of a high concentration of TEMPO mediator. To verify this hypothesis, we proceeded with an additional experiment and checked if the preparative-scale *E. coli*/Lk-ADH Prince-catalyzed bioreduction of **2** was affected by the elevated amount of

TEMPO (41 mg, 0.26 mmol). The results of this trial proved that TEMPO was detrimental to the employed ADH, and thus the bioreduction proceeded less efficiently to afford (R)-(-)-**1** in only 29% isolated yield at 47% conv., but without erosion in enantiomeric excess (>99% ee). However, the subsequent experimental validation also concerned measuring the pH value of the final reaction medium of the one-pot/two-step deracemization procedure. It turned out that the addition of 0.1 M Tris-HCl buffer (pH 7.5) to the concentrated reaction's residue consisting of 50 mM citrate buffer (pH 5.0) gave a mildly acidic mixture with pH 6.2.

To avoid a negative interaction of the acidic medium with *E. coli*/Lk-ADH Prince, we have optimized its pH by a proper dilution of the reaction mixture with 0.1 M Tris-HCl buffer (pH 8.0), leading to a pH adjustment of ca. 7.5. This time, the bioreduction step was not affected by the pH of the medium and the TEMPO mediator, and thus the yield of the whole process improved significantly. Finally, the application of modified reaction conditions of LTV/TEMPO-*E. coli*/Lk-ADH Prince-mediated deracemization of *rac*-**1** resulted in the isolation of (R)-(-)-**1** in 67% yield with 94% ee.

3. Conclusions

In this work, an attempt was made to synthesize optically active lisofylline in both enantiomeric forms (S)-(+)-**1** and (R)-(-)-**1** through a straightforward one-pot/two-step redox-driven chemoenzymatic-deracemization route employing laccase from *Trametes versicolor* coupled with TEMPO as a redox mediator, and the subsequent combination of two recombinant alcohol dehydrogenases (*E. coli*/ADH-A or *E. coli*/Lk-ADH Prince) as biocatalysts for an asymmetric hydrogen transfer bioreduction of the *in situ* generated pentoxifylline (**2**). In this regard, a complex screening of the optimal reaction conditions for (laccase/TEMPO)-mediated non-selective oxidation of racemic lisofylline (*rac*-**1**) was performed to obtain pentoxifylline (**2**) in high 95% conv. and 68% yield. Next, a series of recombinant ADHs were screened in stereoselective reduction of prochiral ketone **2** to afford both enantiomers of LSF. Finally, asymmetric bioreductions were integrated with the elaborated oxidation step. Applying the (LTV/TEMPO)-*E. coli*/ADH-A system afforded (S)-lisofylline in 56% yield and 94% ee, while employing the LTV/TEMPO-*E.*



Scheme 2 Deracemization of racemic lisofylline (*rac*-**1**) catalyzed by *T. versicolor* laccase/TEMPO-alcohol dehydrogenase system.



coli/Lk-ADH Prince system provided (*R*)-lisofylline in 67% yield and 94% ee. One-pot deracemization of *rac*-1 revealed that *E. coli*/Lk-ADH Prince was more sensitive toward TEMPO than *E. coli*/ADH-A, which rationalizes the lower yield in this case. The chemoenzymatic approach presented herein constitutes a valuable extension of the synthetic repertoire to synthesize challenging pharmaceuticals in high enantiomeric enrichment and completes our journey toward non-racemic lisofylline. Although this attempt is the first example of the employment of the LTV/TEMPO-ADH catalytic system toward deracemization of unactivated *sec*-alcohols, further optimization of these cascades and expansion of the substrate scope to understand the full potential of laccase/TEMPO-ADH tandem catalysis is required to make it a direct approach in fruitful biotransformations.

4. Experimental

4.1. Materials and methods

Laccase from *Trametes versicolor* (LTV, 0.66 U mg⁻¹) was purchased from Sigma-Aldrich (Cat. No.: 38429). 2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical (TEMPO) was purchased from Sigma-Aldrich (Cat. No.: 214000). β -Nicotinamide adenine dinucleotide, disodium salt, hydrate, 95+%, reduced form (NADH) was purchased from Across Organics (Cat. No.: 271100010). All other reagents for chemical transformations and product isolation/purification were obtained from commercial sources and used as received without additional pretreatment. Citrate buffer (pH 5, 50 mM) was saturated with molecular oxygen by bubbling it for 30 min prior to being used in the chemoenzymatic oxidation experiments. The chromatographic (GC) analyses were performed using an Agilent Technologies 6890 N instrument equipped with a flame ionization detector (FID) and fitted with an HP-50+ (30 m) semipolar column (50% phenyl-50% methylpolysiloxane); helium (2 mL min⁻¹) was used as carrier gas; retention times (t_R) are given in minutes under these conditions. The GC method conditions for the resolution of the respective compounds *rac*-1, 2, and *rac*-3 are shown in Table S2 (see the ESI[†]). The enantiomeric excesses (% ee) of bioreduction products were determined by HPLC analysis performed on a Shimadzu CTO-10ASV chromatograph equipped with an STD-20A UV detector and/or a Shimadzu Nexera-*i* (LC-2040C 3D) instrument equipped with a photodiode array detector (PAD) using a Chiralpak AD-H (4.6 mm \times 250 mm, coated on 5 μ m grain size silica gel, from Daicel Chemical Ind., Ltd.) chiral column equipped with a respective pre-column (4 mm \times 10 mm, 5 μ m); the HPLC analyses were executed in an isocratic and isothermal (25 $^{\circ}$ C) mode; the method conditions for the resolution of racemic lisofylline (*rac*-1) are shown in Table S3 (see the ESI[†]). Optical rotations ($[\alpha]$) were measured with a PolAAR 32 polarimeter in a 2 dm long cuvette using the sodium D line (λ = 589 nm). ¹H NMR (500 MHz) and ¹³C NMR (126 MHz) spectra were recorded on a Varian NMR System 500 MHz spectrometer. The ¹H and ¹³C chemical shifts (δ) are reported in parts per

million (ppm) relative to the solvent signals [CDCl₃, δ_H (residual CHCl₃) 7.26 ppm, δ_C 77.16 ppm]. Chemical shifts are presented as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br s (broad singlet); coupling constants (J) are reported in hertz. Raw spectroscopic data were processed with ACD/NMR Processor Academic Edition (Product Version: 12.01). Mass spectrometry was recorded on a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, ESI source: electrospray with spray voltage 4.00 kV for FTMS analysis; all samples were prepared by dilution of MeOH (0.5 mL) and additives of mixtures of CH₃CN/MeOH/H₂O (50 : 25 : 25, v/v/v) + 0.5% formic acid (HCOOH) each.

4.2. Synthetic procedures

4.2.1. General procedure for the PCC-mediated oxidation of (*rac*-1). To a solution of lisofylline (*rac*-1, 280 mg, 1.0 mmol) in CH₂Cl₂ (10 mL), pyridinium chlorochromate (PCC, 323 mg, 1.50 mmol) was added portion-wise over a period of 15 min at 25 $^{\circ}$ C. After 12 h of vigorous stirring, an amount of Celite (200 mg) was added, followed by CHCl₃ (10 mL). The heterogeneous slurry mixture was stirred for 5 min, then filtered over a pad of Celite and washed with CHCl₃ (3 \times 10 mL). Evaporation of the filtrate under reduced pressure afforded a crude residue, which was further purified by column chromatography on SiO₂ eluting with a CHCl₃/acetone (90:10 v/v) mixture. The obtained solid was additionally quenched with Et₂O (2 \times 1 mL) and dried under reduced pressure to afford the desired ketone 2 (203 mg, 0.73 mmol, 73%) as a white solid.

3,7-Dimethyl-1-(5-oxohexyl)-3,7-dihydro-1H-purine-2,6-dione (2). Mp 108–111 $^{\circ}$ C (Et₂O) [lit.⁴³ 104–106 $^{\circ}$ C (CHCl₃)]; R_f [CHCl₃/acetone (90 : 10, v/v)] 0.15; ¹H NMR (500 MHz, CDCl₃): δ 1.58–1.70 (m, 4H), 2.12 (s, 3H), 2.48 (t, J = 7.1 Hz, 2H), 3.55 (s, 3H), 3.96 (s, 3H), 3.99 (t, J = 7.1 Hz, 2H), 7.49 (s, 1H); ¹³C NMR (126 MHz, CDCl₃): δ 21.1, 27.5, 29.8, 30.0, 33.7, 40.9, 43.3, 107.7, 141.5, 148.8, 151.6, 155.4, 208.8; IR (Nujol): ν_{max} = 2924, 1660, 1456; FTMS (ESI-TOF) m/z : [M + H]⁺ Calcd for C₁₃H₁₉N₄O₃⁺ m/z : 279.14517, found 279.14504; GC [260 (const.)]: t_R = 9.75 min.

4.2.2. General procedure for the synthesis of silylated lisofylline *rac*-3. To a solution of racemic lisofylline (*rac*-1, 118 mg, 0.42 mmol) in CH₂Cl₂ (3 mL), *N,O*-bis(trimethylsilyl)acetamide (BSA, 342 mg, 1.68 mmol, 415 μ L) was added in one portion, and the reaction mixture was stirred at 40 $^{\circ}$ C for 30 min. Next, the volatile compounds were evaporated under vacuum, and the crude product was purified by SiO₂ column chromatography using a mixture of CHCl₃/MeOH (95:5, v/v) as an eluent to afford the desired silylated lisofylline *rac*-3 (102 mg, 0.30 mmol, 69%) as a white solid.

3,7-Dimethyl-1-{5-[(trimethylsilyl)oxy]hexyl}-2,3,6,7-tetrahydro-1H-purine-2,6-dione (*rac*-3). Mp 119–123 $^{\circ}$ C (CHCl₃/MeOH); R_f [CHCl₃/MeOH (95:5, v/v)] 0.30; ¹H NMR (500 MHz, CDCl₃): δ 0.08 (s, 9H), 1.11 (d, J = 6.1 Hz, 3H), 1.26–1.52 (m, 4H), 1.57–



1.72 (m, 3H), 3.56 (s, 3H), 3.71–3.79 (m, 1H), 3.97 (d, $J = 0.5$ Hz, 3H), 3.99 (s, 2H), 7.48 (d, $J = 0.5$ Hz, 1H); ^{13}C NMR (126 MHz, CDCl_3): δ 0.4, 23.6, 24.0, 28.2, 29.8, 33.7, 39.4, 41.5, 68.6, 107.8, 141.5, 148.9, 151.6, 155.4; IR (Nujol): $\nu_{\text{max}} = 2924$, 1656; FTMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ Calcd for $\text{C}_{16}\text{H}_{29}\text{N}_4\text{O}_3\text{Si}^+$ m/z : 353.20034, found 353.20027; GC [260 (const.)]: $t_{\text{R}} = 7.28$ min.

4.2.3. General procedure for the oxidation of *rac*-1 using the laccase/TEMPO catalytic system in the presence of aerial O_2

4.2.3.1 Method A (screening of co-solvents). In an open-to-air test tube, TEMPO (4.1 mg, 33 mol%) was added to a suspension of racemic lisofylline (*rac*-1, 23 mg, 0.08 mmol, 50 mM final conc.) in a monophasic mixture of oxygen-saturated 50 mM citrate buffer (pH 5)/organic solvent (10–50% (v/v)), for a total volume of 1.6 mL. The mixture was magnetically stirred for a few minutes to dissolve all the reagents, and then the laccase from *Trametes versicolor* (LTv, 7 mg, 4.6 U) was added. Afterward, the reaction mixture was gently stirred (150 rpm) in the presence of aerial O_2 for 16 h at 30 °C. After this time, the product was extracted with EtOAc (2 × 2 mL), and the organic phases were combined, dried over anhydrous Na_2SO_4 , and filtered. Next, the permeate was concentrated under a vacuum, the remaining oil was dissolved in CH_2Cl_2 (200 μL), and *N,O*-bis(trimethylsilyl)acetamide (BSA, 30 μL) was added using a microsyringe in one portion; after 20 min of vortexing the contents of the Eppendorf tube at room temperature, an aliquot of the sample was directly analyzed using GC. For additional data, see also Table 1. Note: a control reaction (blank experiment) without using LTv was performed; however, no obvious reaction was observed.

4.2.3.2 Method B (effect of acetone amount). In an open-to-air test tube, TEMPO (4.1 mg, 33 mol%) was added to a suspension of racemic lisofylline (*rac*-1, 23 mg, 0.08 mmol, 50 mM final conc.) in a monophasic mixture of oxygen-saturated 50 mM citrate buffer (pH 5)/acetone (10–50% (v/v)), for a total volume of 1.6 mL. The mixture was magnetically stirred for a few minutes to dissolve all the reagents, and then the laccase from *Trametes versicolor* (LTv, 7 mg, 4.6 U) was added. Afterward, the reaction mixture was gently stirred (150 rpm) in the presence of aerial O_2 for 96 h at 30 °C. The rest of the procedure was carried out in analogy to method A. For additional data, see also Table 2.

4.2.3.3 Method C (effect of metal ions). In an open-to-air test tube, TEMPO (4.1 mg, 33 mol%) and the respective inorganic salt (1 mM final conc.) were added to a suspension of racemic lisofylline (*rac*-1, 23 mg, 0.08 mmol, 50 mM final conc.) in a monophasic mixture of oxygen-saturated 50 mM citrate buffer (pH 5)/acetone (20% (v/v)), for a total volume of 1.6 mL. The mixture was magnetically stirred for a few minutes to dissolve all the reagents, and then the laccase from *Trametes versicolor* (LTv, 7 mg, 4.6 U) was added. Afterward, the reaction mixture was gently stirred (150 rpm) in the presence of aerial O_2 and/or with additional O_2 purging (bubbling oxygen) for 24 h at 30 °C. The rest of the procedure was carried out in analogy to

method A and method B. For additional data, see Table S1 in the ESI.†

4.2.3.4 Method D (effect of the reaction time and the laccase/TEMPO amount). In an open-to-air test tube, TEMPO (4.1 mg, 33 mol%) and laccase from *Trametes versicolor* (LTv, 7 mg, 4.6 U) were added sequentially every 24 h to a suspension of racemic lisofylline (*rac*-1, 23 mg, 0.08 mmol, 50 mM final conc.) in a mixture of oxygenated 50 mM citrate buffer (pH 5)/acetone (20% (v/v)), for a total volume of 1.6 mL. The reaction mixture was gently stirred (150 rpm) in the presence of aerial O_2 for 48–120 h at 30 °C. The rest of the procedure was carried out in analogy to methods A–C. For additional data, see also Table 3.

4.2.4. General procedure for semi-preparative scale oxidation of *rac*-1 using the laccase/TEMPO catalytic system in the presence of aerial O_2 . In an open-to-air test tube, TEMPO (8.2 mg, 33 mol%) and the laccase from *Trametes versicolor* (LTv, 14 mg, 9.2 U) were added sequentially every 24 h to a suspension of racemic lisofylline (*rac*-1, 46 mg, 0.16 mmol, 50 mM final conc.) in a mixture of oxygenated 50 mM citrate buffer (pH 5, 2.56 mL) and acetone (640 μL). The reaction mixture was stirred with a magnetic stirrer (150 rpm) in the presence of aerial O_2 for 120 h at 30 °C. After this time, the content of the vial was extracted with EtOAc (2 × 4 mL), the organic phases were combined, dried over anhydrous Na_2SO_4 , and filtered. Next, the permeate was concentrated under vacuum, and the remaining oil was subjected to SiO_2 -based column chromatography using $\text{CHCl}_3/\text{MeOH}$ (95:5, v/v) as an eluent, thus affording the desired ketone 2 (31 mg, 0.11 mmol, 68% yield, 95% conv.) as a white solid. The spectroscopic data for 2 were essentially the same as for the chemical standard obtained in the reaction with the PCC reagent (see protocol 4.2.1.). The values of % conv. were assigned using the established protocol employing BSA as a derivatization agent and GC. For additional data, see also Table 4.

4.2.5. General procedure for the analytical-scale studies on stereoselective bioreduction of prochiral ketone 2 with different whole-cell biocatalysts – screening procedure. Each of the lyophilized whole-cell biocatalysts, including wild-type microorganisms and/or *E. coli* cells with overexpressed recombinant ADHs (10 mg), were suspended in the reaction solution (500 μL), containing 20 mM glucose, 0.5 mM NADH, and 10 mM ketone 2 in a mixture of 0.1 M Tris-HCl buffer (pH 7.5)/2-ProH/DMSO (500 μL ; 87.5:10:2.5, v/v/v). In general, biotransformations were conducted in glass vials ($V = 1.5$ mL) without air access for 48 h at 30 °C using a laboratory shaker (250 rpm) in a final volume of 0.5 mL. After this time, each reaction was stopped by extracting the content of the vial with EtOAc (3 × 1 mL), the combined organic phase was dried over anhydrous MgSO_4 , the filtrate was additionally centrifuged (5 min, 6000 rpm), and finally the supernatant was transferred into a separate HPLC vial and concentrated under vacuum. The oil residue in one of the vials was used to determine % conv. by using GC analysis after derivatization of the crude mixture with BSA (see



protocol 4.2.5.1 below), and the other oil residue (2 mg) was re-dissolved in HPLC-grade 2-PrOH (1.5 mL) and analyzed by HPLC on a chiral stationary phase to establish enantiomeric excesses of optically active alcohols. For additional data, see also Table 5. Note: lyophilized cells of the appropriate biocatalysts were rehydrated at first by suspending them in an aqueous buffer solution of 0.1 M Tris-HCl (pH 7.5) (337.5 μ L), NADH (50 μ L) added from a 5 mM stock solution prepared by dissolving the cofactor (3.55 mg) in 0.1 M Tris-HCl (pH 7.5, 1 mL), and in the case of wild-type microorganisms, glucose (50 μ L) was also added from a 0.2 M stock solution prepared by dissolving carbohydrate (36 mg) in 0.1 M Tris-HCl (pH 7.5, 1 mL). After 30 min of incubation of the whole-cell biocatalysts at 30 °C (250 rpm), the reaction was initiated by the addition of ketone **2** (50 μ L) from a 0.1 M stock solution prepared by dissolving substrate **2** (27.8 mg) in 2-PrOH (1 mL) and supplemented with DMSO (12.5 μ L).

4.2.5.1 Derivatization of the samples for GC analyses with BSA as silylation reagent. To a vial containing oil residue after enzymatic reactions, a solution of *N,O*-bis(trimethylsilyl)acetamide (BSA, 15 mg, 71.3 μ mol, 18 μ L) in CH_2Cl_2 (100 μ L) was added in one portion. After 20 min of vigorous vortexing of the reaction mixture at room temperature, an aliquot of the sample was directly analyzed using GC.

4.2.6. General procedure for the semi-preparative scale stereo-complementary bioreduction of **2 using (*S*)-selective *E. coli*/ADH-A or (*R*)-selective *E. coli*/Lk-ADH Prince enzymes.** The respective *E. coli*/ADHs (60 mg) were suspended in 0.1 M Tris-HCl buffer (1.75 mL; pH 7.5) containing NADH (1.42 mg, 1.0 mM final concentration) and preincubated for 30 min at 30 °C. Then, a solution of ketone **2** (45 mg, 0.16 mmol) in 2-propanol (200 μ L, 10% (v/v)) supplemented with DMSO (50 μ L, 2.5% (v/v)) was added to the mixture. The reaction was shaken (250 rpm) at 30 °C for 48 h and then stopped by extraction with EtOAc (3 \times 5 mL). The organic layers were combined and dried over anhydrous MgSO_4 . After filtering off the drying agent and evaporating the volatiles, the crude residue was purified by column chromatography on SiO_2 gel using a mixture of $\text{CHCl}_3/\text{MeOH}$ (95 : 5, v/v) as an eluent. The obtained yellowish solid was further purified by dissolving it in CH_2Cl_2 (0.5 mL) and adding a few drops of EtO_2 until the solution became turbid. Next, the mixture was placed in a freezer and cooled at -18 °C for 30 min until a white solid precipitated. Subsequent filtration of the solid and drying under high vacuum afforded the desired optically active products: (*S*)-(+)-**1** [30 mg, 67% isolated yield, >99% ee, $[\alpha]_{\text{D}}^{24.5} = +7.5$ (*c* 1.00, CHCl_3) {lit.¹³ $[\alpha]_{\text{D}}^{24.0} = +7.5$ (*c* 1.00, CHCl_3)}] in the case of *E. coli*/ADH-A, and (*R*)-(-)-**1** (29 mg, 65% isolated yield, >99% ee, $[\alpha]_{\text{D}}^{24.5} = -5.0$ (*c* 1.00, CHCl_3) {lit.⁹ $[\alpha]_{\text{D}}^{25.0} = -8.75$ (*c* 0.40, CHCl_3)}] in the case of *E. coli*/Lk-ADH Prince.

4.2.7. General procedure for semi-preparative scale deracemization of lisofylline (*rac*-1**) catalyzed by the (laccase/TEMPO)-ADH system.** In an open-to-air test tube, TEMPO (8.2 mg, 33 mol%) and laccase from *Trametes versicolor* (LTV, 14 mg, 9.2 U) were added sequentially every 24 h to a

suspension of racemic lisofylline (*rac*-**1**, 46 mg, 0.16 mmol, 50 mM final conc.) in a mixture of oxygenated 50 mM citrate buffer (pH 5, 2.56 mL) and acetone (640 μ L). The reaction was stirred with a magnetic stirrer (150 rpm) in the presence of aerial O_2 for 120 h at 30 °C. After this time, a small sample of the crude mixture was withdrawn to establish the % conv. (see protocol 4.2.5.1. with BSA above), and the reaction conditions were changed to bioreductive by diluting the concentrated residue (*ca.* 0.5 mL) with 0.1 M Tris-HCl buffer (3.4 mL; pH 7.5 in the case of *E. coli*/ADH-A) or 0.1 M Tris-HCl buffer (3.4 mL; pH 8.0 in the case of *E. coli*/Lk-ADH Prince), 2-PrOH (400 μ L; to achieve 10% (v/v)), and DMSO (100 μ L; to achieve 2.5% (v/v)) and reach 4 mL of the final reaction volume. Next, NADH (2.84 mg; to reach 1.0 mM final conc.) and the respective *E. coli*/ADHs (60 mg) were added, the glass tube was closed, and the reaction mixture was shaken (250 rpm) at 30 °C for 48 h. After incubation, the enzymatic reaction was stopped by extraction with EtOAc (3 \times 5 mL). The organic layers were combined and dried over anhydrous MgSO_4 . After filtering off the drying agent and evaporating the volatiles, the crude residue was purified by column chromatography on SiO_2 gel using a mixture of $\text{CHCl}_3/\text{MeOH}$ (95 : 5, v/v) as an eluent. The obtained yellowish solid was further purified by dissolving it in a small amount of CH_2Cl_2 (0.5 mL) and precipitating it with EtO_2 . After cooling the solution at -18 °C for 30 min, a fine precipitate was filtered off using a Pasteur pipette plugged with cotton wool and dried under a high vacuum to afford the desired optically active products as white solids: (*S*)-(+)-**1** (26 mg, 56% isolated yield, 94% ee) in the case of *E. coli*/ADH-A, and (*R*)-(-)-**1** (30 mg, 67% isolated yield, 94% ee) in the case of *E. coli*/Lk-ADH Prince.

Author contributions

P. B.: conceptualization, methodology, validation, investigation, formal synthesis and analysis, data curation and processing, writing – original draft, writing – review & editing & revision, visualization, supervision, project administration, funding acquisition; A. R.: investigation, formal synthesis and analysis; T. R.: investigation, preparation of biocatalysts; W. K.: writing – original draft, proofreading.

Conflicts of interest

The authors declare no competing financial interest. All authors have approved the final version of the manuscript. There are no conflicts to declare.

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