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Research Article

Multienzymatic Stereoselective Reduction of Tetrasubstituted Cyclic Enones to Halohydrins with Three Contiguous Stereogenic Centers

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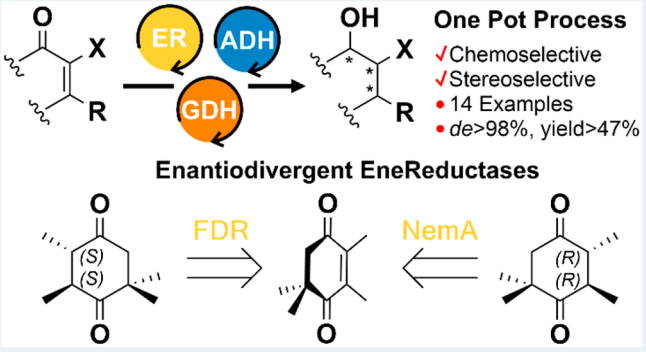
ABSTRACT: The asymmetric hydrogenation of conjugated tetrasubstituted alkenes with transition-metal catalysts is a challenging reaction, especially for substrates bearing a halide substituent. We describe a two-step multienzymatic reduction of a series of α-halo β-alkyl tetrasubstituted cyclic enones, affording halohydrins with three contiguous stereogenic centers, in good yield and with a high stereoselectivity. The reduction is catalyzed by a stereospecific ene-reductase (OYE2-3 or NemA) and a highly enantioselective alcohol dehydrogenase (ADH). The use of two enantiodivergent ADHs allows the control of the diastereoselectivity. The absolute stereochemical configurations of the products have been determined from the analysis of single-crystal structures (Flack's parameter). The enantiomeric excess (*ee*) has been determined by derivatization of the products with (*R*) Mosher's acid. Lastly, we extended our methodology also to a nonhalogenated substrate: the α-methyl ketoisophorone was reduced by two distinct enantiodivergent ene-reductases (flavin mononucleotide- and F_{420}-dependent), affording each enantiomer of the saturated ketone with *ee* > 98%.



KEYWORDS: *ene-reductases, alcohol dehydrogenases, tetrasubstituted enones, chlorohydrins, biocatalysis*

INTRODUCTION

The one-pot stereoselective reduction of CO and C=C double bonds of tetrasubstituted enones **I** is an appealing transformation since it gives secondary alcohols with three contiguous stereogenic centers, that is, **II** (Figure 1a). Surprisingly, its implementation is very recent. It was accomplished through an asymmetric hydrogenation catalyzed by an Ir complex with a chiral phosphine ligand in the presence of *t*-BuONa.[1] Even though selectivity and yields were high, the substrate scope was limited by $R_1 = CO_2Et$. However, if the R_1 substituent is a halogen, it is possible to obtain the halohydrins **III**. Especially interesting would be the stereoisomers of **III** with the halogen substituent trans to the OH group because they can be easily converted to α-alkyl epoxides **IV**, which are synthons that are more versatile than **II**. But, to the best of our knowledge, such a retrosynthesis has never been realized, most likely because the α-haloenones can undergo dehalogenation at typical reaction conditions of the transition-metal-catalyzed hydrogenations.[2]

In addition, it is well-known that the stereoselective reduction of tetrasubstituted C=C double bonds (either isolated or conjugated), catalyzed by transition-metal complexes with chiral ligands, is one of the most challenging reactions of organic chemistry.[3] Good conversions are achievable only by using high H_2 pressures and/or high

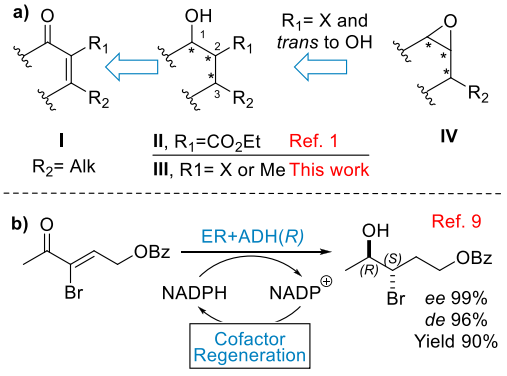


Figure 1. (a) Retrosynthesis of alcohols **II**, chlorohydrins **III**, and epoxides **IV**. (b) Complete reduction of a α-bromo trisubstituted enone catalyzed by enzymes.

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temperatures.[3b,c] Therefore, at harsher experimental conditions, the dehalogenation side reaction should increase.

Completely different is the scenario in the field of biocatalytic reductions.[4] Indeed, enzymes with ene-reductase (ER) activity[5] and enzymes with alcohol dehydrogenase (ADH) activity[6] are able to reduce α-halogenated enones and ketones at mild reaction conditions, and the dehalogenation is usually not observed. Especially the ERs are becoming valuable catalysts in organic synthesis, not only for their use in stereospecific reductions of the C=C double bond conjugated with an electron-withdrawing group (EWGs) but also for their use in enantioselective carbocyclizations[7a,b] and coupling with photocatalysts in multistep processes.[7c,d]

The combination of ER and ADH activities has proven to be effective in the reduction of many prochiral enones and enals. Chiral alcohols with up to two stereogenic centers are usually obtained in good yield and with high stereoselectivity.[8] Recently, we have shown that the multienzymatic reduction of an α-bromo trisubstituted enone proceeded smoothly, affording the corresponding bromohydrin[9] with high optical purity (Figure 1b).

On the other hand, it should be noted that although in the last 2 decades many ERs have been discovered and tested, their substrate scope is restricted to the reduction of trisubstituted conjugated alkenes. Indeed, so far just two tetrasubstituted conjugated alkenes have been reduced.[8e,10] This is likely due to the fact that such substrates are believed to be too sterically hindered[11] to be accepted by most of the ERs.

Nonetheless, in this study we show how ene-reductases can be used as efficient catalysts for the reduction of sterically demanding substrates such as the tetrasubstituted conjugated alkenes **1a**−**1l** (Figure 2). In addition to the canonical flavin mononucleotide (FMN) cofactor dependent ERs (such as the OYEs belonging to the Old Yellow Enzyme family), also the enantiodivergent deazaflavin F_{420}-dependent ERs (FDRs) were investigated.[12] Lastly, we show that ERs coupled with ADHs reduce α-halo tetrasubstituted cyclic enones, affording the corresponding halohydrins in good yield and with high selectivity.

RESULTS AND DISCUSSION

At first, we tested the C=C double-bond reduction of substrates **1a**−**l** with a set of different recombinant ERs. OYE2 and OYE3 from *Saccharomyces cerevisiae* and NemA from *Escherichia coli* were used because they are known for their good performances (selectivity and conversion) with sterically hindered substrates.[8e,10] For regeneration of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor, we used glucose dehydrogenase (GDH) and glucose as the sacrificial cosubstrate.[13] In the Supporting Information the results of the screening can be found (Table S1 and for the best results see Table 1).

Most of the substrates were reduced with good conversions and with a discrete diastereoselectivity (up to *de* > 99% by GC-MS), except for the menadione and the cyclopentenone chloro derivatives, **1h** and **1i**, respectively, which were transformed into the corresponding saturated ketones in a very low yield. For the α-bromo substituted substrates, that is, **1a** and **1k**, we obtained mainly the debrominated saturated products instead of **2a** and **2k** (Scheme 1a). Especially, for the reduction of **1k**, we detected in the reaction mixture also the presence of the maleimide intermediate (see Supporting Information). Hence, probably, as soon as **2a** and **2k** are formed, a spontaneous HBr

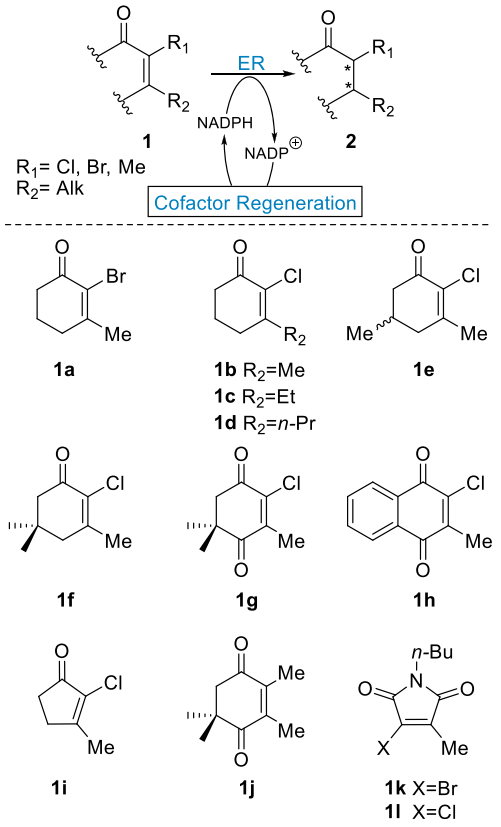

Figure 2. Substrate scope of the C=C double-bond reduction of tetrasubstituted conjugated alkenes.

elimination occurred. The latter intermediates being well accepted by most ERs, they were promptly reduced (Scheme 1a). Moreover, we do not exclude that the formation of dehalogenated products[9,14] could be due to alternative and competitive chemical paths based on ER-catalyzed radical reactions.[7b]

However, it is a fact that the reduction of the chloro analogues, that is, **1b** and **1l**, proceed smoothly, and the side product formation was almost negligible (for **1l** < 5%, by GC-MS). Thus, in view of coupling the two reductive steps on a semipreparative scale (4.0 mmol), we concluded that the chloro derivatives are better substrates than their bromo analogues because the intermediate ketones are more stable at our reaction conditions.

Concerning the reduction of the 2-chloro-3-alkyl cyclohex-2-enones, **1b**−**1d**, the conversion with OYE2 decreased significantly by increasing the alkyl chain length: from 99% for R_2 = Me (**1b**) to 26% for R_2 = *n*-Pr (**1d**). In this regard, Stewart and co-worker observed a similar trend in the OYE1 (similar ER isolated from *Saccharomyces carlsbergensis*)-catalyzed reduction of the alkyl cyclohex-2-enone analogues: from a quantitative conversion for the 3-methylcyclohex-2-enone, the yield dropped down to 18% for the substrate with the *n*-propyl substituent; all products had (*S*)-configuration).[15]

Next, we tested the racemic mixture of enone **1e** bearing a methyl group at the C(5) carbon to see whether the ERs can reduce preferentially one enantiomer over the other, allowing a kinetic resolution.[14b,16] With NemA the conversion was too high (70%), whereas OYE2 and OYE3 gave the product in a yield too low (18% and 7%, respectively). However, in both cases the diastereomeric excess was insignificant because a

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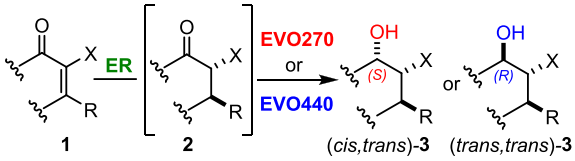
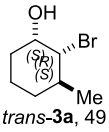
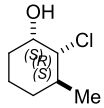
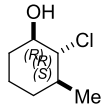
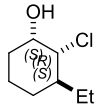
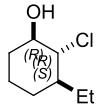
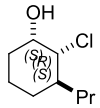
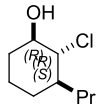
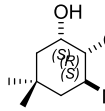
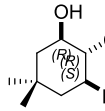
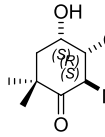
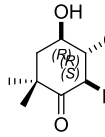
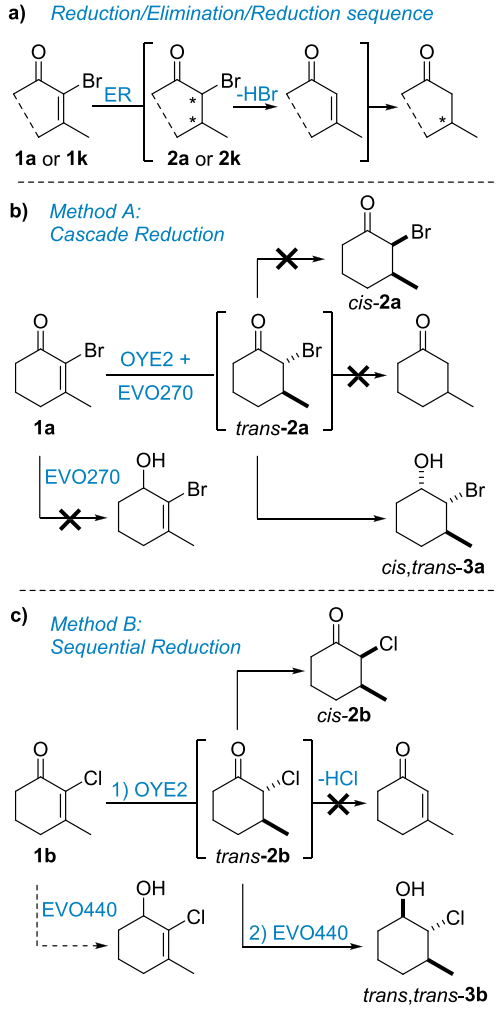
Table 1. Reduction of α-Halo Tetrasubstituted Conjugated Alkenes



Subs.	ER[a] conv. (%),[b] (*de* [%])[c]	Method A: ER + EVO270(*S*)[d] yield (%),[e] (*de* [%])[c]	Method B: 1) ER; 2) EVO440(*R*)[f] yield (%),[e] (*de*[%])[c]
1a	**2a** OYE2 31[g] (91)	 *cis,trans*-**3a**, 49 (>99)	–[g),h)]
1b	**2b** OYE2 99 (92)	 *cis,trans*-**3b**, 83 (>99)	 *trans,trans*-**3b**, 78 (>99)
1c	**2c** OYE2 72 (91)	 *cis,trans*-**3c**, 83 (>99)	 *trans,trans*-**3c**, 78 (>99)
1d	**2d** OYE2 26 (95)	 *cis,trans*-**3d**, 47 (>99)	 *trans,trans*-**3d**, 20 (>99)
1e	**2e** NemA 70[i]	–[b)]	–[b)]
1f	**2f** NemA 72 (99)	 *cis,trans*-**3f**, 84 (>99)	 *trans,trans*-**3c**, 67 (>99)
1g	**2g** OYE2 76 (92)	 *cis,trans*-**3g**, 78 (>99)	 *trans,trans*-**3g**, 58 (>99)
1h	**2h** OYE2-3, NemA –	–	–
1i	**2i** OYE2 <5	–	–
1k	**2k** NemA <5[g]	–	–
1l	**2l** OYE3 73 (92)	–	–

[a]Best ER, 24 h at 30 °C, see Table S1. [b]Conversion by GC-MS, not isolated. [c]By ^{1}H NMR and/or GC-MS. [d]Reaction conditions for Method A: substrate (4.0 mmol), *i*-PrOH as cosolvent (1−2% in volume), ER (OYE2 or NemA, 10−12 mg), GDH (400 U), EVO270 (30−40 mg), $NADP^+$ (20 mg), glucose (6.0 equiv), and pH 7 KPi buffer (50 mM, 100 mL), 24 h at 30 °C. [e]Isolated yield after column chromatography purification. [f]Reaction conditions for Method B: (1) substrate (4.0 mmol), *i*-PrOH as cosolvent (1−2% in volume), ER (OYE2 or NemA, 10−12 mg), GDH (400 U), $NADP^+$ (15 mg), glucose (4.0 equiv), and pH 7 KP_i buffer (50 mM, 100 mL), 12 h at 30 °C, and (2) EVO440 (30−40 mg), glucose (2.0 equiv), $NADP^+$ (5 mg), and GDH (400 U), 24 h at 30 °C. [g]We observed the dehalogenated side product. [h]Product in trace, not isolated. [i]Complex mixture of diastereoisomers.

Scheme 1. (a) Proposed Reduction/Elimination/Reduction Reaction Sequence in the ER Catalyzed Reductions of α-Bromo Substituted Substrates; (b) Example of Cascade Reduction (Method A); (c) Example of Sequential Reduction (Method B)



complex distribution of the four possible diastereoisomers was detected (by GC-MS).

The NemA-catalyzed reduction of the chloro derivative of isophorone, that is, **1f**, gave **2f** in a good yield of 72%, whereas both yeast OYEs failed. Interestingly, the doubly activated chloro-ketoisophorone **1g** was instead reduced with OYE2 affording the ketone **2g** together with the levodione side product (8:2), in an overall conversion of 76% and with a good *de* of 92%.

For the enzymatic reduction of the carbonyl group to be coupled to the ER-catalyzed step, we selected two commercially available ADHs, EVO270 and EVO440, having stereoselectivity pro (*R*) and pro (*S*), respectively. The cofactor regeneration (NADPH) for the ER + ADH multienzymatic process was the same as that applied to the ER-catalyzed biotransformations. Conversions and chemo-

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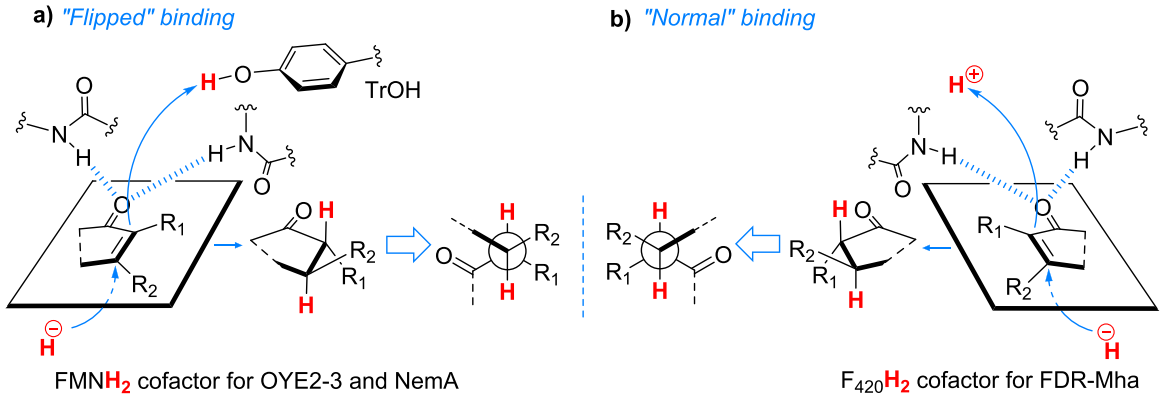

Figure 3. Reduction mechanism and possible binding modes of tetrasubstituted cyclic enones in the ER catalytic site: (a) "flipped" binding for OYE-type ERs and (b) "normal" binding for FDRs.

selectivity data of the screening are available in the Supporting Information (Table S3). Interestingly, we found that EVO270 was sufficiently chemoselective to be added to the reaction mixture together with the ER (Method A: [ER + ADH] cascade reduction, Scheme 1b) because the carbonyl enone reduction, affording the allylic alcohol byproduct, was in most cases negligible. In contrast, EVO440 could be added only after that most of the starting material was consumed, usually no earlier than after 12 h (Method B: [(1) ER; (2) ADH] sequential reduction, Scheme 1c).

After having identified the best ER−ADH combinations, we repeated the biotransformations on a higher scale (4.0 mmol). The yield, after column chromatography purification, and the diastereomeric excess (by ^{1}H NMR or GC-MS) are shown in Table 1. We found that the simultaneous addition of ER (OYE2 or NemA) with EVO270 (Method A) shows a cascade effect because the conversion of the starting material improved substantially with respect to that achieved using the ER standalone (Table S1). In this regard, remarkable was the cascade reduction of **1a** with OYE2 and EVO270 (Method A, Scheme 1b), in which the CO of the reactive intermediate **2a**, as soon as it was formed, was reduced with a reaction rate sufficiently high to minimize the formation of the side product. Indeed, the *cis,trans*-**3a** bromohydrin was isolated at a yield of 49% and with an excellent *de* (>99% by ^{1}H NMR). In contrast, the reduction of the same substrate with Method B gave mainly the 3-methylcyclohexan-1-ol and the *trans,trans*-**3a** isomer was present just in traces (Table 1).

The reduction of the 4-ketoisophorone to give the (*R*)-levodione followed by the regio- and enantioselective reduction of the less hindered carbonyl group has been the object of intense research because the (4*R*,6*R*)-actinol, produced by a chemo-enzymatic process,[17] is a key precursor for the synthesis of several carotenoids of industrial relevance.[18] In this regard, it is noteworthy that the 5-chloro derivatives of actinol, that is, the chlorohydrins *trans,trans*-**3g** and *cis,trans*-**3g**, were obtained with our methodology in good yield and with high optical purity. These findings open new routes to oxygenated carotenoids such as crustaxanthin or to apocarotenoids such as the 3,4-dihydroionone.[19]

The NMR characterization of both diastereoisomers of alcohols **3** allowed us to assign their relative stereochemical configurations (*trans,trans* or *cis,trans*) simply by measuring the *J* coupling constants of the C*H*−X proton signal (see Supporting Information).

The outcomes on the relative stereochemistry gave further evidence that the OYEs-catalyzed reductions proceed by formal addition of H_2 to the C=C double bond with *anti* stereospecificity.[20] The hydride, from the reduced flavin cofactor ($FMNH_2$), attacks the enone's β carbon, forming the carboanion. The latter is then protonated by the acidic hydroxyl group of a tyrosine residue, in the *transoid* position with respect to the $FMNH_2$, resulting in a two stepwise 1,4-addition (Figure 3a). This reaction mechanism has been elucidated for the reduction of conjugated tri- and disubstituted alkenes by a combination of computations[21] and not trivial experiments such as deuterium labeling, rate constant measures, and enzymatic mutations.[22] Instead, using cyclic tetrasubstituted enones is considerably easier to determinate the stereochemical course (by ^{1}H NMR coupling constants). Indeed, we could easily demonstrate that NemA (just 36% sequence identity with OYE2) has the same stereospecificity of the two OYEs (Figure 3a).

The absolute stereochemical configurations of *trans,trans* chlorohydrins **3b** and **3c** were estimated from the Flack parameter obtained from the single-crystal X-ray diffraction model (Figure 4a−c); for more details see the Supporting Information.[23] Unfortunately, for most of the *cis,trans* series, no crystals could be obtained. The only exception is **3g**, to which the (4*S*,5*R*,6*S*) configuration was assigned again by X-ray analysis (Figure 4c).

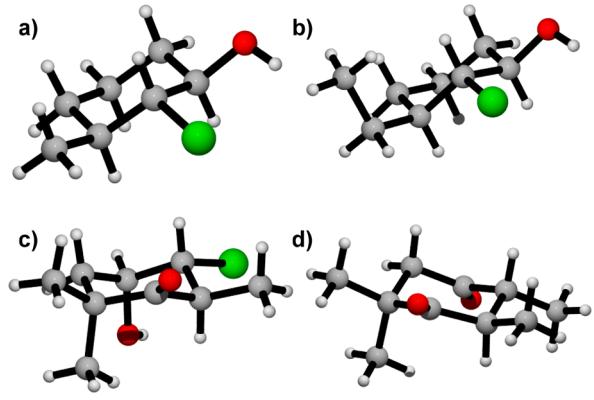

Figure 4. Molecular geometries determined from single-crystal X-ray diffraction: (a) (1*R*,2*R*,3*S*)-**3b**; (b) (1*R*,2*R*,3*S*)-**3c**; (c) (4*S*,5*R*,6*S*)-**3g**; (d) (5*R*,6*R*)-**2j**. All crystals were grown in pentane.

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In a biocatalytic transformation, the absolute stereochemical configuration of the product depends mainly on how the substrate is oriented into the active site of the enzyme. Historically, two possible geometries of binding for OYE-type ERs have been defined: "flipped" or "normal" binding modes (Figure 3). Hence, by taking into account the absolute configurations of chlorohydrins (*trans,trans*)-**3b**,**3c** and (*cis,-trans*)-**3g**, determined from the X-ray crystal structures, we concluded that the OYE2 and NemA arrange the substrates **1b**, **1c**, and **1g** by a "flipped" binding mode, and that EVO270 and EVO440 have actually enantioselectivity pro (*S*) and pro (*R*), respectively.

Although the high diastereomeric excesses achieved can be explained only if both reductive steps are highly stereoselective, we still had some doubts about the optical purity of the products, especially for the *cis,trans* diastereoisomers, of which the absolute configuration was indirectly assigned. Since we were not able to synthesize the racemic mixtures, necessary for the setup of the chiral GC or HPLC analysis,[24] we opted for the derivatization of the *cis,trans* chlorohydrins **3a**−**3f** with the (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride affording Moscher's esters ((*R*)-MTPA-**8a**−**8f**, see Supporting Information).[25]

Both ^{1}H and ^{19}F NMR spectra of **8a**−**8f** esters gave further support to our initial assumption about the high stereoselectivity of the multienzymatic process since the diastereomeric excess was always high (*de* > 98%, see Supporting Information).

Lastly, we show how the bioreductions can be very effective also with non α-halogenated tetrasubstituted enones. Thus, according to our ongoing research program on stereoselective synthesis of chiral flavors and fragrances, and their olfactory evaluation,[26] we focused our interest on the reduction of the tobacco flavoring agent α-methylketoisophorone[27] **1j** to give **2j** (Scheme 2).

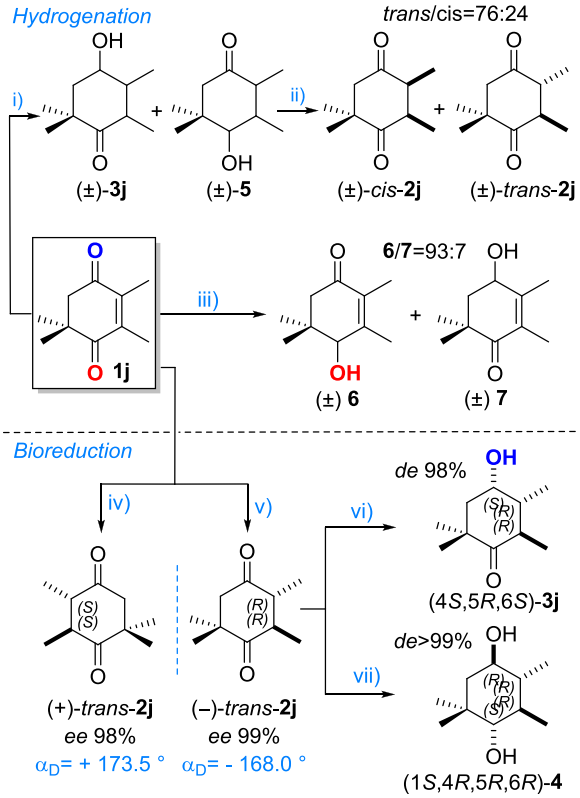
The direct synthesis of *cis*-**2j** isomer by *syn* stereospecific hydrogenation failed (Pd/C in MeOH at 0 °C)[28] since a nonregioselective reduction of the carbonyl groups occurred as well (a very complex diastereomeric mixture of the regioisomeric hydroxyketones **3j** and **5** was detected by GC-MS, Scheme 2). Thus, in the attempt to improve the chemoselectivity toward the formation of *cis*-**2j**, we carried out the reaction at a lower temperature (−10 °C) and for a shorter reaction time. However, at these new reaction conditions, we obtained the allylic alcohol **6** with a quite good regioselectivity (**6**/**7** = 93:7, by ^{1}H NMR). Between the two carbonyl groups, the one that was mostly reactive was, to our surprise, the one more sterically hindered.[29]

Finally, *cis*-**2j** was obtained by oxidation of the mixture of hydroxyketones **3j** and **5**. Although the conversion with the Dess-Martin periodinane was quantitative, the diastereomeric excess was quite disappointing (*trans*/*cis* = 76:24 by ^{1}H NMR).

Less problematic was the enzymatic reduction of **1j** with NemA, which afforded *trans*-**2j** (X-ray structure shown in Figure 4d, $\alpha_D = -168.0°$ in CH_2Cl_2) in a high yield of 84% and with a very good diastereo- and enantioselectivity (*de* > 98% by GC-MS of the crude material and *ee* = 99% by chiral GC after column chromatography, see Supporting Information).

Recently, deazaflavin cofactor (F_{420})-dependent ene-reductases (FDRs) were shown to exhibit opposite stereoselectivity[12] to that of most FMN-dependent ERs,[30] including NemA. This is explained by a "normal" binding mode of

Scheme 2. Stereodivergent Reduction of 2j[a]



[a]Reaction conditions: (i) H_2, Pd/C cat., MeOH, 0 °C to rt, 99% yield; (ii) DMP, CH_2Cl_2, 0 °C to rt, yield 99%; (iii) same of (i), −10 °C to rt, 70% yield; (iv) FDR-Mha, FGD, $F_{420}H_2$ cofactor, glucose-6-phosphate in *tris*·HCl buffer at 24 °C; (v) NemA, GDH, glucose, $NADP^+$ cofactor in KPi buffer at 30 °C, 84% yield; (vi) Method A: the same of (iv) together with EVO270, 90% yield; (vii) Method B: the same of (iv) followed by the addition of EVO440, 82% yield.

substrates **I** into the FDR enzyme active site (Figure 3b). Three different FDRs were tested and found to convert quantitatively **1j** with a high selectivity (Table S3). FDR from *Mycobacterium hassicum* (FDR-Mha) was used for further experiments. Reduction of **1j** with FDR-Mha produced, when compared with NemA, the other enantiomer of the *trans* diketone, that is, (5*S*,6*S*)-**2j** (α_D = +173.5° in CH_2Cl_2), in a good yield of 80% and with a high stereoselectivity (*de* > 99% and *ee* > 98% by chiral GC).

Unlike the OYEs, FDRs require the reduced $F_{420}H_2$ cofactor, which was conveniently regenerated during the biotransformation by means of a F_{420}-dependent glucose-6-phosphate dehydrogenase (FGD, from *R. jostii*), and using an excess of glucose-6-phosphate as sacrificial cosubstrate.[31]

Then the optically pure (−)-diketone was submitted to the carbonyl reduction with each of the two enantiodivergent ADHs. Differently from the Pd/C-catalyzed hydrogenation, EVO270 reduced the less hindered carbonyl group, affording the hydroxyketone (4*S*,5*R*,6*S*)-**3j**, whereas the reduction with EVO440 was not regioselective at all since we isolated the diol (1*S*,4*R*,5*R*,6*R*)-**4**. However, for both reductions, yield and stereoselectivity were more than satisfactory (Scheme 2).

CONCLUSION

In summary, we have shown that ERs can reduce efficiently the C═C double bond of sterically demanding substrates such as

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ACS Catal. 2020, 10, 13050−13057

the α-chloro tetrasubstitued enones, disubstitued maleimides, and the α-methyl ketoisophorone, usually with good yield and high stereoselectivity. In this case, the use of an enzymatic approach is not just a "green" alternative to the transition-metal-catalyzed hydrogenations, but it remedies a typical weakness of the latter in reducing sterically hindered substrates, especially those bearing halide substituents, which easily undergo dehalogenation.

In addition, the combination of OYE-type ERs with ADHs, in a cascade process or in a sequential reduction, allowed the one-pot stereoselective preparation of α-chlorohydrins/alcohol with three contiguous stereogenic centers, and a diol with four stereogenic centers, in good yield and with high stereoselectivity.

Lastly, this study also shows the benefit of using the newly discovered F_{420}-dependent ERs (FDRs), which complement nicely the available OYE-type ERs by their opposite enantioselectivity. The access to two distinct enantiodivergent ERs allowed the synthesis of both enantiomers of a chiral flavor.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.0c04097.

Preparation and characterization of starting materials and products of enzymatic reactions, extensive enzymatic screenings, optimization of individual and multi-enzymatic protocols; copies of GC chiral analyses and ^{1}H and ^{13}C NMR spectra (PDF)

MS and single crystal difraction data (CIF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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