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Bacterial monooxygenase mediated preparation of nonracemic chiral oxiranes: study of the effects of substituent nature and position

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Abstract—Monooxygenation of styrene derivatives using recombinant *E. coli* biocatalyst is an efficient way to prepare the corresponding oxiranes. The electronic and geometric effects of the ring substituents are described and show the relaxed specificity of the enzyme and its high stereoselectivity.
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1. Introduction

The importance of homochiral oxiranes as building block in the preparation of synthons or auxiliaries is well known.[1] Recently, the biocatalytic approach has gained more and more importance as an alternative to chemical catalysis due to its environmental compatibility and low cost.[2] There are two main biocatalytic approaches to homochiral oxirane production: racemate resolution by, mainly, hydrolases,[3] and direct double bond oxygenation by monooxygenases.[4–6] The enzymes of this last class are difficult to isolate and purify, and the cofactor [NAD(P)H] regeneration is difficult to optimize. Nevertheless, at least one group has developed sound processes for oxirane production at good scale.[2] In contrast, not much work has been devoted to the potential of enzyme recognition. This is in part due to the previously cited difficulty of the enzyme isolation; in part due to the need to work with reactive compounds (oxiranes), whose purification and characterization can present problems.

Our group has been involved in the use of whole cell bioconversions for many years and, despite the encountered difficulties, we have studied the biotransformation of several compounds.[7] In the course of this study and with the support of the results of other groups we have reached various conclusions concerning the bioconversions. The first, and most important, factor affecting the reaction is definitely the need to perform the experiment under two-phase conditions. In fact, the substrates are often volatile and toxic to the cell, the products are unstable, volatile, and toxic. Consequently, effort has been spent on the search for good (nontoxic, stable, water immiscible, etc.) organic phases[8] and for optimal reaction conditions (phase ratio, substrate concentration, stir speed, etc.).

Herein we describe our new results concerning enzyme recognition towards some substituted styrenes.

2. Results

In the course of our research, we have used a recombinant *E. coli* JM109 (pTAB19)[9] biocatalyst that was realized by inserting the styrene monooxygenase from *P. fluorescens* ST, a strain selected from the soil for its ability to grow on styrene.[10] This biocatalyst was used for the transformation of a preliminary set of styrene derivatives and showed a good recognition selectivity.[11] The initial compound set was randomly chosen to test different substrate classes: ring substituted styrenes, chain substituted styrenes, cyclic compounds. Herein we have analyzed four complete sets of ring monosubstituted styrenes to study their electronic and

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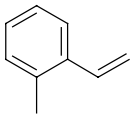
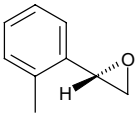
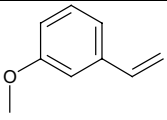
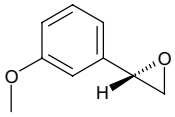
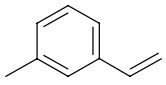
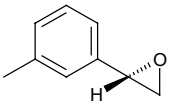
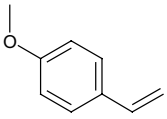
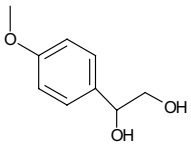
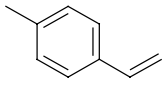
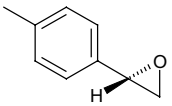
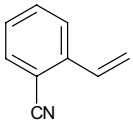
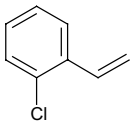
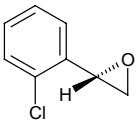
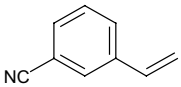
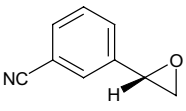
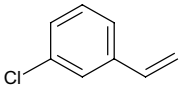
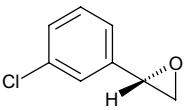
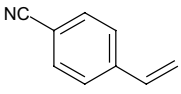
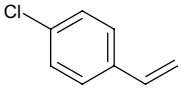
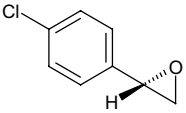
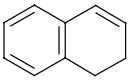
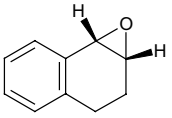
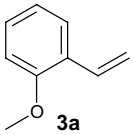
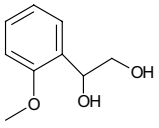
position effects, which were chosen as representative of alkylated, halogenated, electron donating, and electron withdrawing groups. The reactions were performed under two principal reaction conditions: in the first the substrates are dissolved into isooctane, in the second they are dissolved into isooctane–isopropyl ether mixtures; in all cases the organic phases contained either 10 g/L of substrate, or a saturated lower concentration, in the perspective of providing a sufficient but nontoxic amount of substrate. This new experimental approach produced two interesting effects: first, we noted an increase in the efficiency of the bioconversion also for the already studied compounds (styrene, 4-Me styrene); second, we can transform more substrates. All the experiments were performed with a comparison bioconversion of styrene; in this way, we wanted to render as much as possible the results independent of the biocatalyst current activity. As a consequence, the enzyme recognition is expressed as a percentage of the performance on styrene. The results are presented in Table 1. It is clear that the recognition is influenced by both the substituent position and nature.

The first very interesting remark concerns the unexpected monotonic increase of the yield along the series *ortho*, *meta*, *para*. In contrast with the common elec-

Table 1. Substrates, products, and relative activity, of the bioconversion of styrene derivatives

Substrate	Product	Relative activity[a] (%)	Ee[b]	Substrate	Product	Relative activity[a] (%)	Ee[b]
 **1a**		46	56	 **3b**		138 25% diol[c]	>95
 **1b**		101 (142)	>95	 **3c**		222	n.d.[c]
 **1c**		130 (153)	>95	 **4a**	—	0	
 **2a**		60	>95	 **4b**		55	>95
 **2b**		107 (100)	>95	 **4c**	—	0	
 **2c**		145	>95	 **5**		43 (184)	>95
 **3a**		55	n.d.[c]				

[a] Relative activity = [specific activity of substrate (i)]/[specific activity of styrene] × 100; Specific activity of (i) = mmoles of products formed in 1 h by 1 g of cells (dry cell weight) in 1 L of broth, as calculated from the correlation line of the first conversion hours. Number in parentheses refer to the results reported by Hollmann et al.[12]

[b] Enantiomeric excess is measured by chiral GLC.

[c] n.d. = not determined; the racemic diols derive from the chemical hydrolysis of the corresponding epoxides and are not representative of their enantiomeric excess.

tronic effects *ortho*-substituted compounds do not show an influence greater than the corresponding *meta*-substituted compounds. This result is logically correlated to the simultaneous geometry effect, that seems to be stronger in the *ortho* position, lowering the corresponding yields. The yield ratio is roughly 1:2 comparing *ortho*- and *meta*-substituted compounds, whilst *meta*- and *para*-substituted substrates show a smaller ratio (~1:1.3). The position effect is so strong that when considering the expected electronic effect (OMe > Me ~ Cl > CN) in the *ortho*-substituted compounds we can note a quasi-null influence; the only exception being the CN group that is deactivating enough to completely stop the transformation.

Looking at the *meta*-substituted compounds we observe a more expected result; the electronic effect is not very strong and all the compounds are transformed in similar amounts. Again the CN group depresses the reactivity, even if, in this case, we can isolate the bioconversion product. Finally, the *para*-substituted compounds show the expected influence of the group electronics, with a clear preference for the most electron donating group (OMe) (ratio 1.5–1.7). We can also include in this topic the surprisingly higher yield presented by the *para*-substituted compounds in comparison to styrene. In many cases, the 'native' substrate is transformed by enzymes at a higher rate than the other substrates; this is not true in our case, all the *para* compounds, CN-substituted excluded, give very good conversion rates (from 1.3 to 2.2 higher than styrene). Even *meta*-substituted compounds show rates comparable to styrene. This fact is in disagreement with both our previous experiments[11] and the results reported in the literature;[2] nevertheless, all the present experiments give this result. It is interesting to note that the obtained activity ratios are in partial agreement with the initial rate ratio reported by Hollmann et al.[12] If we consider that they were using an isolated environment (enzyme and metal recycling of FAD cofactor) we can speculate that the reaction conditions that we are presently using are independent of the transport mechanism; of course, this fact requires an experimental proof.

A second interesting point concerns the isolated products in the case of activated aromatic rings (MeO-substituted on the *ortho* and *para* position). Here, the only isolated product is the 1,2-diol that clearly derives from the spontaneous hydrolysis of the corresponding epoxide. This is confirmed by the complete lack of enantiomeric excess, by a control experiment performed on the isolated oxiranes in the absence of the biocatalyst, and by the great difficulty that we experienced also in the isolation of the chemically prepared epoxides (reaction that is performed in the presence of water). In the performed experiments we operated using an isooctane/isopropyl ether mixture that could solubilize good substrate amounts and that, we hoped, could extract most of the product from the water phase. This unfortunately did not happen and the product hydrolyzed. Nevertheless, the production rate is still representative. Having learnt of this possibility, we always checked also the water phase looking for the existence of possible diols. In addition, we detected the production of small amounts of reduced compounds (2- and4-OMe 2-phenyl ethanol), due to a reductive activity of the cells.

The third point concerns the enantiomeric excesses of the products. As in most of our previous experiments we expected to isolate nearly enantiopure oxiranes; this was the case for all compounds except *o*-methyl styrene. The corresponding oxirane shows a 75:25 enantiomer ratio, which represents the lowest excess we have ever determined: We do not have a clear rationalization for this. Excluding the possibility of a nonenzymatic racemization of the compound, we can think only of a relatively low enzyme facial recognition. In order to get a further piece of data concerning the problem of *o*-substituted substrates, we performed the bioconversion using 1,2-dihydronaphthalene as a substrate representative of alkyl *ortho* substituents with low steric hindrance, but with completely removed rotation freedom. The result confirms the lower transformation rate, in agreement with all the previous data, but, in contrast to the methyl substituent, the ee was high, as usual.

3. Conclusion

Using whole cell transformations of styrenes by the cloned SMO (styrene mono oxygenase) from *P. fluorescens* ST we could prepare several chiral epoxides with different enantiomeric excess, most of them practically enantiopure. In addition, we could partly elucidate the enzyme recognition capability, obtaining interesting and unexpected results. Work is in progress to determine the correct conditions to prepare also those oxiranes that showed too high reactivity towards hydrolysis.

4. Experimental

4.1. Analytical methods

Reaction progress was followed by gas chromatography (DANI 86.10 gas chromatograph with FID) on a Chrompack Cp-Sil 8CB column ($T = 100 \pm 150\,^{\circ}C$ at $15\,^{\circ}C\,min$, splitless injection) with the appropriate internal standard (dodecane or hexadecane). At intervals, 2 mL samples were taken from the reaction emulsion. The two phases were separated by filtration. The water phase was followed by HPLC on a Merck/Hitachi (L-6200) system connected to a UV-detector set at 230 nm on a (C18 Hibar Lichrosorb 50334, 5 μm, 25 cm) column with 50:50 CH_3CN/water. The absolute configuration of biocatalytically prepared (*S*)-styrene oxide could be proven via comparison with commercially available, enantiopure (*S*)-styrene oxide (Aldrich). Based on the analogous chromatographic behavior of racemic mixtures, we assume the (*S*)-configuration for all epoxides. Enantiomeric excesses were measured using a Chrompack ChiralDex-CB column by comparison to synthetic racemic mixture. Optical rotations were measured with a Perkin Elmer 341 polarimeter. NMR spectra were recorded on a Bruker AC 200 (^1H NMR at

200 MHz). All signals are expressed as ppm down field from tetramethylsilane. All the compounds show spectra in agreement with the literature data.

4.2. Biocatalyst preparation and bioconversion procedure

Biocatalyst *E. coli* JM109 (pTAB19) was prepared by adding 1 mL of an overnight LB culture in 100 mL M9 medium containing: glucose 10 mM; thiamine 0.05 mM; kanamycin 50 μg/mL; IPTG (isopropyl-β-D-thiogalactopyranoside) 1 mM as inducer; incubated overnight on a shaker at 30 °C. After the growth, OD 1.2–2.0 (λ 600 nm), the cells were separated by centrifugation (10,000 rpm, 4 °C) and added to 70 mL M9 medium containing glucose 10 mM on a shaker at 30 °C; the bioconversion was started by adding the substrate (concentration of 10 g/L in the organic phase) dissolved into 30 mL of isooctane (or isooctane/isopropyl ether 9:1 mixture when appropriate). The transformation was carried out at 30 °C.

4.3. General procedure for chemical preparation of alkenyl substrates

Methyl-triphenylphosphonium iodide (20 mmol), potassium carbonate (25 mmol), the aldehyde (20 mmol), were added to 20 mL of dioxane containing a small amount of water; the solution has been refluxed under agitation for 5–7 h, or until the substrate disappears. The mixture was then filtered to eliminate the salts, dried on Na_2SO_4 and evaporated at reduced pressure very cautiously to avoid material loss. The products were purified by silica gel chromatography to eliminate triphenylphosphine oxide and used in the bioconversions.

4.4. General procedure for chemical preparation of racemic epoxides

The olefin (2 mmol) in CH_2Cl_2 (10 mL) was mixed with an equal amount of water containing $NaHCO_3$ (1 g); to this solution was cautiously added 3-chloroperbenzoic acid (2.2 mmol). The reaction mixture was stirred at rt for 2.5 h, or until the substrate disappears. Afterwards it was washed with aqueous Na_2SO_3 (1.3 g in 10 mL) for 20 min; the aqueous phase was then extracted with CH_2Cl_2 (2×10 mL). The organic phases were washed with $NaHCO_3$ (2×25 mL) and with water. The CH_2Cl_2 phase was dried over anhydrous $MgSO_4$ and evaporated at reduced pressure.

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