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Microbiological transformations. Part 39: Determination of the regioselectivity occurring during oxirane ring opening by epoxide hydrolases: a theoretical analysis and a new method for its determination

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Abstract

In the course of this work we have devised new equations as well as a new method allowing for the total determination of the regioselectivity occurring during biohydrolysis of a racemic epoxide by an epoxide hydrolase. This determination is achievable by simply studying the racemic epoxide as a substrate. The results showed that, depending on the enantioselectivity (E value) and the regioselectivity involved, the absolute configuration as well as the enantiopurity of the residual epoxide and of the formed diol appear to be highly variable. For a specific enzyme/substrate couple, the yield and enantiopurity of the less reactive (remaining) epoxide — and thus the possibility to prepare it in enantiopure form — exclusively depend upon the enzyme enantioselectivity. On the other hand, the ee of the formed diol (eep) depends upon the enantioselectivity and on the *regioselectivity* of the oxirane ring opening. A theoretical analysis based on the material balance, as well as several practical examples, are provided to illustrate the various possibilities of such biohydrolyses.

1. Introduction

The value of epoxides and/or of their corresponding vicinal diols as synthetic intermediates for the total synthesis of optically active drugs emphasizes the need to obtain these compounds in a high state of enantiomeric purity. In addition to chemo-catalytic methods,[1–3] the use of epoxide hydrolases (EHs) is a new, very actively emerging strategy for the access to enantiopure epoxides or vicinal diols.[4,5] These diols can be either cyclised back to the corresponding epoxide or used as cyclic sulfates or sulfites,[6] known as

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being highly valuable epoxide-like building blocks. The intimate mechanism involved in the opening of epoxides by mammalian EHs has been recently determined by Amstrong et al.[7] and by Hammock et al.[8] These authors have shown, from single turnover experiments conducted in ^{18}O-enriched water, that the oxygen atom incorporated into the product, though ultimately derived from water, is proximally derived from the enzyme by way of an ester intermediate formed via an *anti* opening of the oxirane ring by an aspartic carboxylate group.

As a general feature, most biocatalyzed reactions are enantioselective, due to the asymmetric nature of enzymes. Thus, their E value (enantioselectivity ratio) can be conveniently evaluated by applying the well known equations previously defined by Sih et al.[9] using reactions catalyzed by lipases. However, in the case of EH catalyzed hydrolyses, the problem appears to be more complicated because an epoxide can be attacked at *both* carbon atoms of the oxirane ring, moreover with different kinetics. Furthermore, the regioselectivity of this attack can be different from one enantiomer to the other. Thus, whereas the ee of the residual epoxide — obtained at a given conversion ratio — exclusively depends upon the E value, the ee of the formed diol depends upon *two* combined factors, i.e. the enantioselectivity *and* the regioselectivity of the oxirane ring opening.[10] This observation leads to the conclusion that Sih's equations implying the ee of the product *cannot* be used in most cases.

From a practical point of view, the accurate analyses of such reactions lead, in numerous cases, to quite puzzling results as far as the stereochemical evolution of the reaction (i.e. variation of the diol ee over the reaction time) is concerned. Various cases have already been described indicating that the nature of the substituent (alkyl or aromatic), as well as the substitution of the epoxide, play an important role in the regioselectivity of the hydrolysis, and thus on the stereochemical outcome of the reaction. For monosubstituted epoxides, it was observed that the regioselectivity was strongly dependent upon the enzyme origin as well as upon the type (alkyl or aromatic) of the substituent borne by the epoxide. Thus, for monoalkyl epoxides, mammalian microsomal[11,12] and cytosolic[13,14] EHs attack occurs very preferentially at the terminal carbon atom while, for aromatic epoxides (such as styrene oxide for example), a lack of regioselectivity was observed with rabbit soluble EH.[14] As far as microbial EHs are concerned, only scarce results are available presently, but it seems that enzymes from bacterial origins[15] would in general exhibit a regioselectivity comparable to the one of mammalian mEHs, whereas fungal EH[16] could show a variation of regioselectivity from one strain to another. Interestingly, for α/β disubstituted oxiranes, it was shown that the regioselectivity may even differ for each enantiomer, and was also dependent upon the relative *cis* or *trans* configuration of the substituents.[17–19] Thus, several cases of enantioconvergency, resulting from an almost total switch of regioselectivity from one enantiomer to the other, were recently described, essentially for *cis*-substituted epoxides hydrolyzed by EHs from bacteria,[18] fungi,[10] plants[20] and mammals.[14,21] Finally, for *gem*-disubstituted[22,23] and trisubstituted[23] epoxides, the regioselectivity was usually shown to be totally on the less sterically hindered carbon atom.

Owing to these facts, an accurate characterization of a reaction performed by an EH obviously necessitates the determination of not only the *enantio*selectivity, but also the *regio*selectivity of the reaction. Up to now, two main methods have been used to reach this goal. The first one consists of carrying out ^{18}O labeling experiments, using either ^{18}O labeled substrates or ^{18}O labeled water. This approach has been employed to determine the regioselectivity for several epoxides, using EHs from either bacterial,[15] fungal,[16] insect,[24] plant[25] or mammalian[26] origin. The second method consists of performing separately the biohydrolysis of both enantiopure enantiomers.[27] The determination of the ee and absolute configuration of the formed diol then allows one to deduce the regioselectivity of water incorporation. This technique, however, requires the preliminary synthesis of both enantiomers in enantiopure form, an approach which, depending on the substrate, might be quite tedious or even impossible.

We describe here a new, accurate and straightforward method allowing for the determination of this

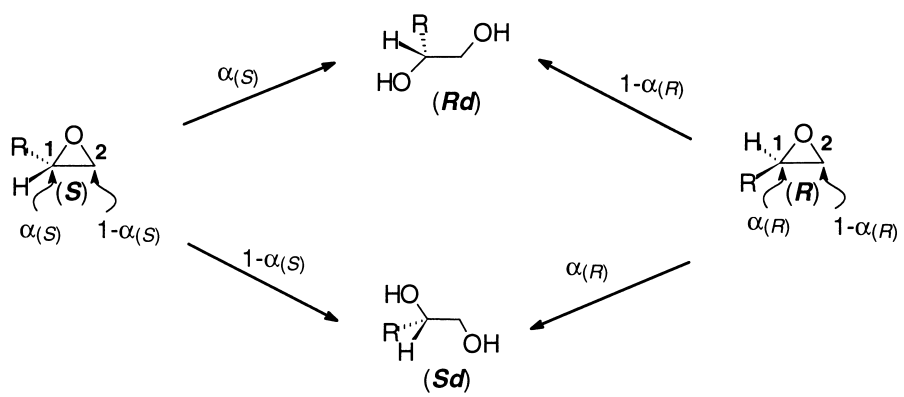
regioselectivity, simply by starting from the racemic epoxide as a substrate. Based on the theoretical development presented in this paper, this approach also allows an understanding of the different possible evolutions of such biocatalyzed reactions. This will be illustrated using results we have observed in the course of our work, aimed at the study of the scope and limitations of the EH catalyzed hydrolyses on various substrates.

2. Results

In the course of our studies, we have achieved a large 'matrix-like' exploration of the scope and limitations related to the possible use of epoxide hydrolases for the synthesis of various enantiopure epoxides. These biohydrolyses have been carried out using enzymatic extracts from various fungi as well as a human sEH prepared using a baculovirus-infected insect cell's overexpression system.[28] The method of preparation of these enzymatic extracts, as well as the experimental conditions used to perform these biohydrolyses and the determination of the absolute configurations of the epoxides and diols, will be published elsewhere.

2.1. Theoretical approach

On the basis of the results we have obtained studying the mechanism of EH catalyzed hydrolyses using fungal EHs,[16] it is reasonable to postulate that an *anti* opening is implied for fungal EHs, similar to the process described in the literature for mammalian EHs. Scheme 1 represents such a hydrolysis which, as shown, can in principle lead to the formation of both enantiomers of the diol from each of the epoxide enantiomers, if the biohydrolysis is not regioselective.[29]



Scheme 1.

In this scheme, the regioselectivity of the hydrolysis will be defined by the new parameters $\alpha_{(S)}$ and $\alpha_{(R)}$, called 'regioselectivity coefficients'. Thus, $\alpha_{(S)}$ represents the fraction of (1*S*)-epoxide attacked at the C_1 carbon atom, and therefore the fraction of (1*R*)-diol formed by hydrolysis of the (1*S*)-epoxide. Similarly $\alpha_{(R)}$ represents the fraction of (1*R*)-epoxide attacked at the C_1 carbon atom, and therefore the fraction of (1*S*)-diol formed by hydrolysis of the (1*R*)-epoxide. Therefore, it must be stressed that a value of α=0.5 means that *no* regioselectivity is observed, whereas *total* regioselectivity leads to either α=0 or 1.

A general theoretical analysis of the evolution of such a reaction, based on the material balance, can be described using the following equations.

$$R_d = [R_0 - R](1 - \alpha_{(R)}) + [S_0 - S]\alpha_{(S)} \quad (1)$$

$$S_d = [R_0 - R]\alpha_{(R)} + [S_0 - S](1 - \alpha_{(S)}) \quad (2)$$

$$c = \frac{[R_d + S_d]}{[R_0 + S_0]} \quad (3)$$

$$\overline{eep} = \frac{1}{c}\left[\frac{[S - R]}{[R_0 + S_0]} + \frac{2\alpha_{(S)}[S_0]}{[R_0 + S_0]} - \frac{2\alpha_{(R)}[R_0]}{[R_0 + S_0]} - \frac{2\alpha_{(S)}[S]}{[R_0 + S_0]} + \frac{2\alpha_{(R)}[R]}{[R_0 + S_0]} + \frac{[R_0 - S_0]}{[R_0 + S_0]}\right] \quad (4)$$

$$[R] = \tfrac{1}{2}(1 - c)[R_0 + S_0](1 - \overline{ees}) \quad (5)$$

$$[S] = \tfrac{1}{2}(1 - c)[R_0 + S_0](1 + \overline{ees}) \quad (6)$$

$$\overline{eep} = \alpha_{(S)} - \alpha_{(R)} + \frac{\overline{ees}(1 - \alpha_{(S)} - \alpha_{(R)})(1 - c)}{c} \quad (7)$$

In these equations, the variables are defined as follows:

$[S_0]$, $[R_0]$	Initial concentration of (1*S*)- and (1*R*)-epoxide
[S], [R]	Concentration of the residual (1*S*)- and (1*R*)-epoxide
$[S_d]$, $[R_d]$	Concentration of the formed (1*S*)- and (1*R*)-diol
$\overline{ees}$	Algebraic value of the epoxide ee: $\overline{ees} = \frac{[S-R]}{[S+R]}$
$\overline{eep}$	Algebraic value of the formed diol ee: $\overline{eep} = \frac{[R_d-S_d]}{[S_d+R_d]}$
c	Conversion ratio
$\alpha_{(S)}$, $\alpha_{(R)}$	Regioselectivity coefficients ($1 \geq \alpha_{(S)}, \alpha_{(R)} \geq 0$)

According to the absolute configuration of the enantiomer which is preferentially hydrolyzed and to the regioselectivities of the opening for each enantiomer, four different 'ideal' cases can thus be observed: (a) residual (*R*)-epoxide, formation of diol (*R*); (b) residual (*R*)-epoxide, formation of diol (*S*); (c) residual (*S*)-epoxide, formation of diol (*R*); (d) residual (*S*)-epoxide, formation of diol (*S*). It is to emphasize that, very often, the cases observed experimentally are in fact a combination of these 'ideal' cases, thus leading to quite complicated figures for the stereochemical evolution of the hydrolysis (see below). In order to establish a general equation which could be used for each one of these four cases, algebraic values of the ee ($\overline{ees}$ and $\overline{eep}$)[30] have been used. From Eqs 1–3 a new equation (Eq. 4) can be deduced, allowing the ee of the formed diol ($\overline{eep}$) to be expressed in terms of the conversion ratio (c), the concentration of each enantiomer of the residual epoxide and the regioselectivity coefficients $\alpha_{(S)}$ and $\alpha_{(R)}$. Similarly, the concentration of each enantiomer of the residual epoxide can be expressed in Eq. 5 and Eq. 6 as a function of their ees and the conversion ratio (c). Combination of Eqs 4–6 led to Eq. 7 which represents the hydrolysis of a racemic epoxide (R_0=S_0). A simplification of this equation is obtained for c=1 (total conversion), the ee of the 'final' diol being at this stage equal to the difference of the two regioselectivity coefficients $\alpha_{(S)}$ and $\alpha_{(R)}$.

In theory, the use of these equations could allow the determination of the $\alpha_{(S)}$ and $\alpha_{(R)}$ values from a combination of two sets of points, using the experimentally determined values of c, ees and eep for each of these experiments. However, in order to obtain a satisfactory accuracy, it is in practice preferable to perform five to six experiments, at relatively different conversion ratios, and to achieve the $\alpha_{(S)}$ and $\alpha_{(R)}$ determination using a curve-fitting computer program.[31] The well known Sih's equation, allowing for the determination of the E value *versus* the conversion ratio and the ees, has been used throughout our work.[9,10]

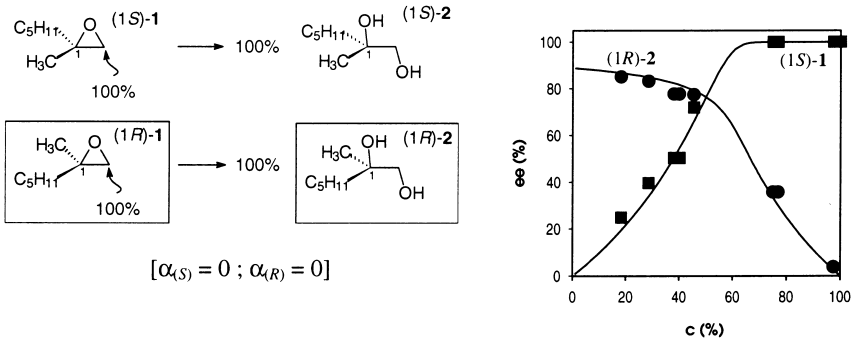


Fig. 1. Biohydrolysis of (±)-**1** catalyzed by a soluble enzymatic extract of *Aspergillus niger*

2.2. Applications

From a theoretical point of view, different cases can be observed starting from a racemic epoxide, since the preferential attack can occur either at the same carbon atom for both enantiomers, or at one carbon atom for one enantiomer, but on the other one for its antipode. This may lead to three different cases: (1) total regioselectivity on the same carbon atom; (2) partial regioselectivity, i.e. attack at both carbon atoms with identical or different proportions, which is obviously the most general case and can lead to various outcomes depending on the regioselectivity and on the proportion of attack at each of the two carbon atoms; and (3) total, but opposite, regioselectivity, i.e. total attack at one carbon atom for one enantiomer and at the other carbon atom for its antipode. Thus, as far as the stereochemical evolution of the reaction (i.e. the value of eep) is concerned, the behavior observed experimentally may very often lead to quite puzzling figures as illustrated below. We will present, for each of them, an explanation of these behaviors based on the use of our $\alpha_{(S)}$ and $\alpha_{(R)}$ regioselectivity coefficients.

2.3. Total regioselectivity on the same carbon atom ($\alpha_{(S)}=\alpha_{(R)}=0$ or 1)

Such a case has been observed, for example, during biohydrolysis of *gem*-1-methyl-1-pentyloxirane-**1** by a crude extract of the fungus *Aspergillus niger* (Fig. 1).[32] This reaction led to the residual (*S*)-epoxide and to the formed (*R*)-diol. Application of Sih's equation led to E=16, and application of Eq. 7 afforded the following regioselectivity coefficient values: $\alpha_{(S)}=\alpha_{(R)}=0$. These results indicate that (a) this reaction was enantioselective and that (b) the attack occurred, for both enantiomers, at the same (less substituted) carbon atom. Obviously in this case, the stereochemical evolution followed the classical scheme, where the ee of the product (formed diol) decreased during the reaction to reach a 0 value for c=1 ($\alpha_{(S)}-\alpha_{(R)}=0$) whereas the ee of the remaining epoxide increased up to 100%. Similar examples have been previously described for some other *gem*-substituted oxirane rings bearing two alkyl groups.[22,23]

2.4. Partial regioselectivity ($\alpha_{(S)}$ and $\alpha_{(R)}\neq 0$ or 1)

As pointed out previously, the above-mentioned case led to an eep equal to zero at completion of the reaction. This is in fact a classical result for biocatalyzed resolutions. In the course of our studies, we have however observed some more complex cases where the formed diol was *not* racemic ($\alpha_{(S)}-\alpha_{(R)}\neq 0$) at a total conversion ratio (c=1). Moreover, different outcomes were obtained where we could observe three different evolutions of the eep: (a) decrease of the formed diol ee during biohydrolysis; (b) increase

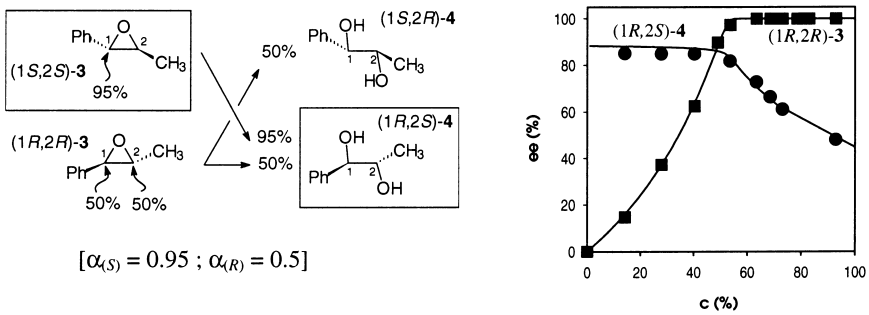


Fig. 2. Biohydrolysis of (±)-**3** catalyzed by a soluble enzymatic extract of *Aspergillus terreus*

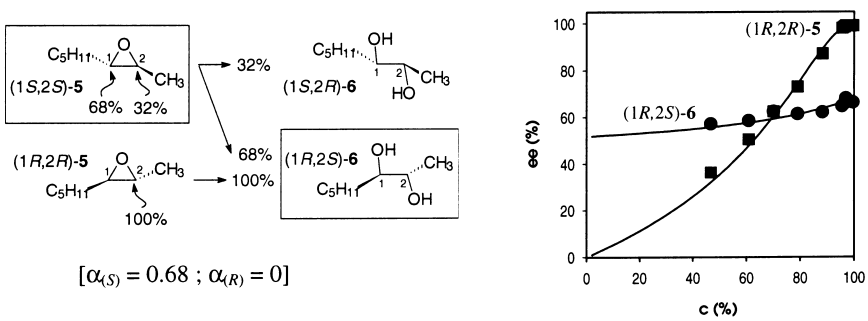


Fig. 3. Biohydrolysis of (±)-**5** catalyzed by a soluble enzymatic extract of *Syncephalastrum racemosum*

of the eep during biohydrolysis; and (c) initial decrease, followed by an increase, of eep. An example of each of these three cases is described below.

2.4.1. Decrease of the eep during biohydrolysis

Such a feature was observed during biohydrolysis of racemic *trans*-2-methyl-1-phenyloxirane **3** by the enzymatic extract from *Aspergillus terreus* (Fig. 2). In this example, the (1*S*,2*S*)-enantiomer was hydrolyzed more rapidly than its antipode, the (1*R*,2*S*)-diol being formed with an ee of approximately 90% until the conversion rate reached about 50%, but decreased later on to about 45% ($\alpha_{(S)}-\alpha_{(R)}$=0.45). Calculation of the E, $\alpha_{(S)}$ and $\alpha_{(R)}$ parameters led to values of E=70; $\alpha_{(S)}$=0.95 and $\alpha_{(R)}$=0.5. This thus indicates that (a) this biohydrolysis was highly enantioselective and (b) it presented an almost total regioselectivity at the benzylic carbon atom for the (1*S*,2*S*)-enantiomer, whereas the (1*R*,2*R*)-antipode showed a regioselectivity shared between both carbon atoms. To observe such an evolution, the regioselectivity of the more reactive enantiomer must be better than that of the less reactive enantiomer. In the opposite case, i.e. if the regioselectivity at the less reactive enantiomer is higher than the regioselectivity on the more reactive one, the following case will be observed.

2.4.2. Increase of the formed diol ee during biohydrolysis

We have observed this case during biohydrolysis of *trans*-2-methyl-1-pentyloxirane **5** by the fungus extract *Syncephalastrum racemosum* (Fig. 3). Indeed, a modest enantioselection in favor of the (1*S*,2*S*) enantiomer was found (E=3) which was opened with a shared regioselectivity ($\alpha_{(S)}$=0.68). On the other hand, the less reactive (1*R*,2*R*) enantiomer was opened with a total regioselectivity at the C_2 carbon atom ($\alpha_{(R)}$=0). Therefore, the ee of the formed diol increased over the course of the reaction, to reach a value of 68% ($\alpha_{(S)}-\alpha_{(R)}$=0.68) after completion of the hydrolysis (c=1).

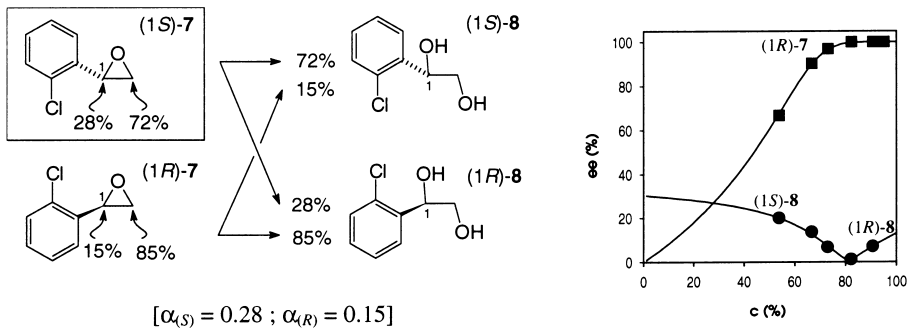


Fig. 4. Biohydrolysis of (±)-**7** catalyzed by human sEH

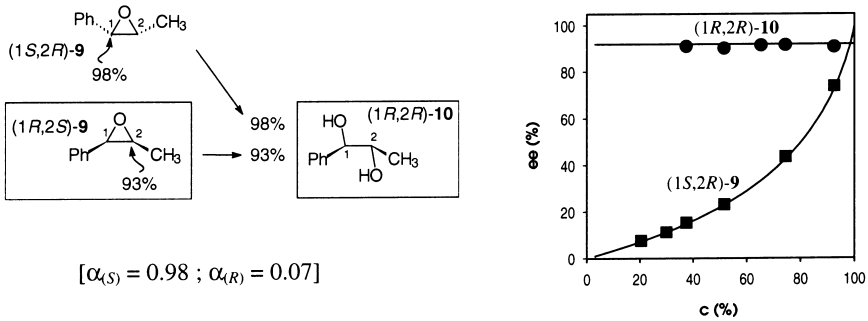


Fig. 5. Biohydrolysis of (±)-**9** catalyzed by a soluble enzymatic extract of *Aspergillus terreus*

2.4.3. Initial decrease, followed by an increase, of eep

Over the course of such a reaction, the absolute configuration of the formed diol switches from one to the other, depending on the conversion ratio. For example, biohydrolysis of *ortho*-chloro-phenyloxirane **7** by a human sEH (calculated E value=7.4) led to the evolution shown in Fig. 4. In this case, the enzyme preferentially attacked both enantiomers at the same (terminal) carbon atom, however with different proportions. Moreover, the faster hydrolyzed (*S*)-enantiomer showed only a moderate regioselectivity ($\alpha_{(S)}$=0.28), whereas the slower reacting (*R*)-antipode showed a higher regioselectivity ($\alpha_{(R)}$=0.15). This led to the preferential formation of the (*S*)-diol at a conversion ratio of lower than 80%, and to a predominance of the (*R*)-product later on. At the end of the reaction, the ee of the formed diol was 13% ($\alpha_{(S)}-\alpha_{(R)}$=0.13).

2.5. Enantioconvergent biohydrolysis ($\alpha_{(S)}$=1, $\alpha_{(R)}$=0 or $\alpha_{(S)}$=0, $\alpha_{(R)}$=1)

Such a case has been obtained during biohydrolysis of *cis*-2-methyl-1-phenyloxirane **9** by the fungal extract from *Aspergillus terreus* (Fig. 5), which proved to be only poorly enantioselective (E=1.9). Thus, the (1*R*,2*S*)-enantiomer was attacked preferentially at C_2 ($\alpha_{(R)}$=0.07), and its (1*S*,2*R*)-antipode at C_1 ($\alpha_{(S)}$=0.98). Interestingly in this case, the ee of the formed diol remained almost constant throughout the course of the reaction. In such a case, the reaction is partly enantioconvergent, thus leading to a diol which shows a high ee (eep=91%). In theory, an enantiopure diol could thus be obtained, with a 100% theoretical yield, if the regioselectivities were opposite and total for each enantiomer.

2.6. Limitations

One limitation of the use of our approach is the fact that Eq. 7 cannot be applied to determine the $\alpha_{(S)}$ and $\alpha_{(R)}$ values if the reaction is not enantioselective (E=1). This is due to the fact that, in this particular case, only the $\alpha_{(S)}-\alpha_{(R)}$ value can be determined. One illustration of such an example was the biohydrolysis of styrene oxide by the enzymatic extract obtained from the fungus *Cunninghamella elegans*, where the two enantiomers were hydrolyzed at the same rate (E=1). In order to determine the $\alpha_{(S)}$ and $\alpha_{(R)}$ values, we therefore had to perform separately the biohydrolysis of the two enantiomers. The results obtained showed that the regioselectivity was identical for both of them ($\alpha_{(S)}=\alpha_{(R)}=0.27$). As a consequence, the ee of the formed diol remained equal to 0 over the entire course of reaction.

3. Conclusion

In the course of this work we have devised new equations allowing for the easy and total determination of the regioselectivity occurring during biohydrolysis of a racemic epoxide by an epoxide hydrolase. This determination is achievable by simply studying the racemic epoxide as a substrate. The results we have obtained during this study showed that such a reaction can be a relatively complex process as far as the stereochemical evolution of the reaction is concerned. Indeed, depending on the enantioselectivity (E value) and the regioselectivity involved, the absolute configuration as well as the enantiopurity of the residual epoxide and of the formed diol, appear to be highly variable. For a specific enzyme/substrate couple, the yield and enantiopurity of the less reactive (remaining) epoxide — and thus the possibility of preparing it in enantiopure form — exclusively depend upon the enzyme enantioselectivity (E value). On the other hand, the ee of the formed diol (eep) depends upon the enantioselectivity and on the regioselectivity of the oxirane ring opening. Several examples have been provided to illustrate the various possibilities of such biohydrolyses.

Acknowledgements

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29. To simplify the presentation, this diagram represents the case of the biohydrolysis of a monosubstituted epoxide, but it remains valid for any differently substituted epoxide. In this case, and in order to keep homogeneity, the oxirane ring is numbered with the convention that the carbon atom C_1 is the one bearing the 'bulkier' substituent.
30. Due to this convention, the ees will be positive if the (1*S*)-epoxide is the slow reacting enantiomer, whereas it will be negative if it is the (1*R*)-epoxide. Similarly, the eep will be positive if the (1*R*)-diol is formed preferentially, whereas it will be negative if it is the (1*S*)-diol.
31. The regioselectivity coefficients were calculated by non-linear regression of Eq. 7 using the commercial Jandel software Sigmaplot® (Marquardt–Levenberg algorithm).
32. By convention, the epoxide enantiomer which is framed is the faster hydrolyzed one and the diol enantiomer framed is the one formed in excess.