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Tetrahedron: *Asymmetry* 15 (2004) 1157–1161
Tetrahedron: *Asymmetry*

Enzymatic resolution of (±)-glycidyl butyrate in aqueous media. Strong modulation of the properties of the lipase from *Rhizopus oryzae* via immobilization techniques

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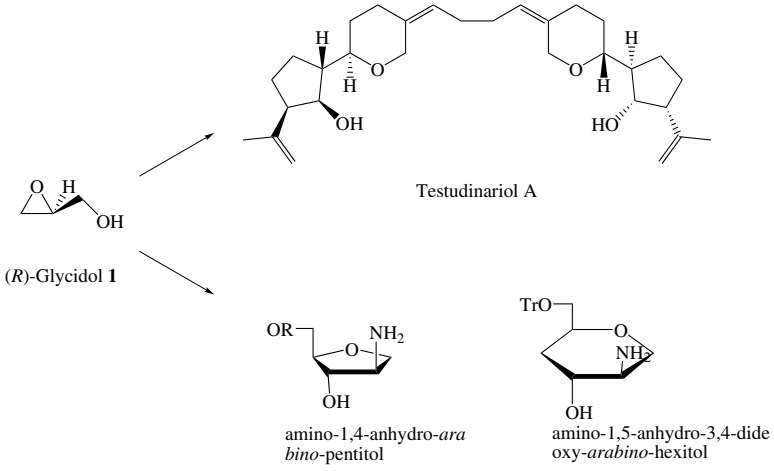
Received 19 January 2004; revised 17 February 2004; accepted 2 March 2004

Abstract—The enantioselectivity of the lipase from *Rhizopus oryzae* for the small yet highly interesting (±)-glycidyl butyrate have been greatly modulated by the use of different immobilization techniques. Thus, the enzyme immobilized by interfacial adsorption on an octyl-agarose support presented a very low enantioselectivity ($E = 2$). This value could be improved up to an $E = 17$ by covalently immobilizing the enzyme on the cyanogen bromide agarose. However, the best results were achieved by immobilizing the enzyme via ionic adsorption on supports coated with dextran sulfate, with an E value of 51 (ee >99% at 55% conversion).

1. Introduction

Enantiomerically pure epoxy alcohols and their esters are very attractive intermediates for the preparation of optically active beta-blockers and a wide range of other products.[1] In this way, (*R*)-glycidol **1** has been used as a synthon in the synthesis of (+)-testudinariol A[2] (Scheme 1). This compound is a structurally unique triterpene alcohol and thought to be a defensive allomone of *P. testudinarius*, because it is ichthyotoxic against the fish, *Gambusia affinis*. Another example is the use of (*R*)-**1** as the starting material in the synthesis of enantiomerically pure amino-anhydro-alditols[3] (Scheme 1). These are a class of compound used as monosaccharide



Scheme 1. Drugs synthesized from (*R*)-glycidol.

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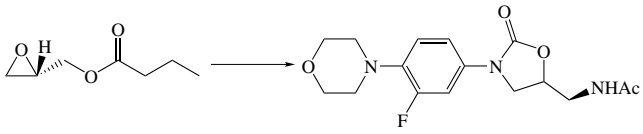
doi:10.1016/j.tetasy.2004.03.003

mimetic structures and amino acid surrogates,[4] as well as the precursor of several organic molecules. In this sense, it has been used as the key intermediate in the synthesis of some natural products,[5] protease inhibitors,[6] and isonucleosides.[7] Furthermore, (*R*)-glycidyl butyrate **2** has been used to introduce a stereogenic center in the synthesis of Linezolid[8] (Scheme 2). This substrate is currently marketed for the treatment of multidrug resistant Gram-positive infections such as nosocomial, community-acquired pneumonia, and skin infections. In this manner, the resolution of glycidyl ester derivatives has constituted an important target in biotransformations.[9] For instance, an enzymatic process has been described using crude porcine pancreas lipase for the resolution of (±)-glycidyl butyrate,[10] process now performed on a multitonne scale,[11] although it was necessary to reach high conversions to obtain adequate enantiomeric excesses. In recent years, the resolution of this compound has been tried with many enzymes acting as biocatalysts.[9,10,12] However, it has been very difficult to find an enzyme, which presents high enantiomeric excess against this compound, probably due mainly to its small size, which reduces any steric hindrance to the adsorption to the active center of the enzyme.



(*R*)-Glycidyl Butrate **2** Linezolid

Scheme 2. (*R*)-Glycidyl butyrate as the key intermediate in the synthesis of Linezolid.

Recently, it has been reported that some good results have been obtained using lipases produced by *Rhizopus oryzae*.[12] Herein, we report an improvement on those results using a new strategy based on the use of different immobilization techniques involving different areas of the enzyme surface, giving a different rigidity to the enzyme structure or generating different microenvironments surrounding the enzyme to alter the exact shape of the open form of lipase. This strategy is denominated as 'conformational engineering' and has successfully been used in the modulation of the behavior of other lipases.[13] This technique has demonstrated that the properties of the same lipase immobilized on different supports could be completely different.

This strategy is based on the drastic conformational changes that lipases undergo during catalysis. In aqueous media, they exist mainly in a closed and inactive conformation,[14] where the active site is secluded from the reaction medium by an oligopeptide chain named as 'lid', in a certain equilibrium with an open and active conformation, where the lid is displaced permitting the accessibility of substrates to the active site. However, upon exposure to a hydrophobic interface, the lipase is adsorbed onto it provoking a conformational change shifting the equilibrium to the open form, a phenomenon known as Interfacial Activation.[15]

2. Results and discussion

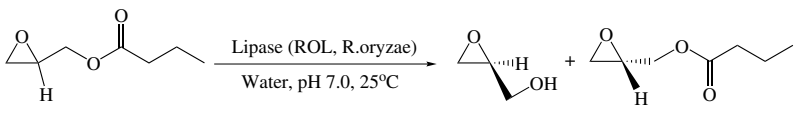
The resolution of (±)-**2** catalyzed by different immobilized purified preparations of a lipase from *R. oryzae* (ROL) in aqueous media at 25 °C and pH 7 was performed (Scheme 3). The lipase was first immobilized by interfacial activation on a hydrophobic support (octyl-agarose).[16] This strategy of immobilization showed very low enantioselectivity value ($E = 2$) with an enantiomeric excess (ee) of 25% at 50% conversion (Table 1). When the lipase was immobilized on agarose activated with cyanogen-bromide (CNBr), where the enzyme was attached mainly by the terminal amino group, ROL showed an increase in enantioselectivity ($E = 17$) with 77% ee at 50% conversion (Table 1). Most interestingly, this lipase, after immobilization by ionic adsorption on amino-Sepabeads coated with very large dextran sulfate (DS) presented an even higher E value ($E = 51$). These results show that ROL can present very different selectivities toward this compound, depending on the immobilization strategy used.

Table 1. Resolution of (±)-**2**[a] catalyzed by different immobilized preparations[b] of ROL

Entry	Support	Time (h)	Conversion (%)	Ee (%)[c]	*E* value[d]
1	Octyl-agarose	0.5	50	25	2
2	CNBr-agarose	3	50	77	17
3	DS-Sepabeads	6	50	89	51

[a] The substrate concentration was 10 mM.
[b] The immobilized preparations of ROL contained 1 mg protein[17]/g support.
[c] Enantiomeric excess (ee) was determined by Chiral HPLC.
[d] Enantioselectivity (*E*) was calculated by using the equation resulted by Chen et al.[18]

Figure 1 shows the evolution of the enantiomeric excess ee (%) of substrate and product versus conversion in the hydrolytic reaction catalyzed by ROL immobilized on DS or octyl-agarose supports. The enantiomeric excess



(±)-**2** (*R*)-**1** (*R*)-**2**

Scheme 3. Enzymatic resolution of (±)-**2** catalyzed by *R. oryzae* lipase.

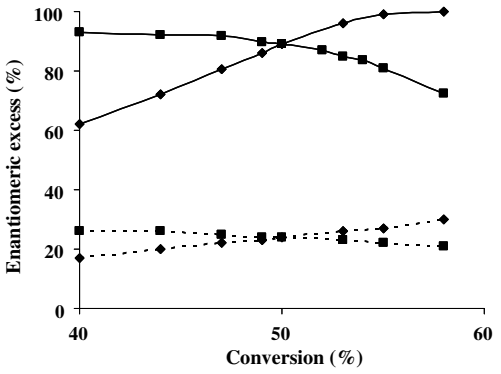


Figure 1. Evolution of enantiomeric excess (ee) versus conversion in the hydrolysis of (±)-**2** catalyzed by different ROL immobilized preparations. Ee of the remaining ester (*R*)-**2** (◆), ee of the release acid (*R*)-**1** (■). DS-ROL preparation (—), octyl-ROL (- - -).

of the remaining ester in the reaction catalyzed by octyl-ROL preparation remained low even at high degrees of conversion, showing an ee of 30% at 58% conversion. Nevertheless, after adsorption of the lipase on amino-Sepabeads coated with dextran sulfate, the enzyme was much more selective toward the (*S*)-enantiomer, obtaining >90% ee at conversions slightly higher than 50%; thus, >99% ee was achieved at 55% conversion, conditions where enantiomerically pure (*R*)-**2** can be obtained. Furthermore, (*R*)-**1** could also be obtained with high ee, by stopping the reaction at 44% conversion (>90% ee of product).

These results compete with those published with lipase from porcine pancreas[10] and improve upon those using this lipase.[12] Moreover, this biocatalyst can be used in at least 30 reaction cycles while maintaining more than 95% of the catalytic activity.

3. Conclusion

In conclusion, by using a suitable immobilization technique (adsorption on dextran sulfate coated Sepabeads), an enzymatic resolution of glycidyl butyrate has been found with >99% ee at 55% conversion catalyzed by a lipase from *R. oryzae*.

4. Experimental

4.1. General

The lipase from *R. oryzae* (ROL) was from Fluka. Octyl-agarose 4BCL and cyanogen bromide (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Amino-Sepabeads were kindly donated by Resindion Srl (Mitsubishi Chem Coorp, Milan, Italy). Dextran sulfate (DS) (Mr 5000), *p*-nitrophenyl propionate (*p*NPP) were from Sigma. (±)-Glycidyl butyrate was kindly donated by Dr. Terreni (University of Pavia) (Milan, Italy). Other reagents and solvents used were of analytical or HPLC grade.

4.2. Enzymatic activity assay determination

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of 0.4 mM *p*NPP in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. One international unit of *p*NPP activity is defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of *p*NPP per minute (IU) under the conditions described above.

4.3. Purification of ROL

Commercial lipase extract was dissolved at 25 mg extract/mL in 5 mM sodium phosphate, then submitted to gentle stirring for 2 h at 4 °C and pH 7, and lastly centrifuged at 12,000 rpm for 30 min.

The lipase preparation was then offered to octyl-agarose following the procedure previously described[16] (100 mg extract per g of support). Periodically, the activity of suspensions and supernatants were assayed using the *p*NPP assay. In order to desorb the enzyme, the adsorbed lipase preparations were washed with 1% Triton X-100 in 5 mM sodium phosphate buffer at pH 7 and 4 °C.

4.4. Immobilization of lipases on different supports

Three different immobilized preparations were produced following the procedures previously described:

- Interfacial activation of the enzyme on octyl-agarose[16] as described above.
- Immobilization on CNBr-activated support using the protocol from Pharmacia (at pH 7).
- Ionically adsorbed lipase on Sepabeads support with amino groups coated with dextran sulfate (DS) (anionic microenvironment surrounding large areas of the protein). The immobilization of the protein was performed at pH 5 and 25 °C.

In all cases, enzyme loading was 1 mg protein/mL of support (that is approx. 1–2% of the maximum load) in order to prevent diffusion problems and more than 95% of the lipase becoming immobilized on all supports offered. The immobilization was followed by the assay described above. Protein concentration was determined by the Bradford method.[17]

4.5. Enzymatic hydrolysis of ester

The activities of different ROL immobilized preparations on the hydrolysis reaction of compound **2** were

performed with the addition of 0.01 g of immobilized preparation to 10 mL of 10 mM of substrate at pH 7 and 25 °C.

During the reaction, the pH value was maintained at a constant using a pH-stat Mettler Toledo DL50 graphic. The degree of hydrolysis was analyzed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450). For these assays, a Kromasil C_{18} (25×0.4 cm) column was used, mobile phase acetonitrile–10 mM ammonium phosphate buffer at pH 2.95 (35:65, v/v) at 1.5 mL/min and UV detection was performed at 225 nm.

4.6. Determination of enantiomeric excess and enantioselectivity

The enantiomeric excess (ee) of the remaining ester was determined on the organic phase obtained by extracting 0.2 mL of aqueous phase with 0.2 mL hexane and analyzed by Chiral Phase HPLC. The column was a Chiracel OD (10 μm 250×4.6 mm), the mobile phase an isocratic mixture of isopropanol and hexane (2:98, v/v) at a flow of 0.4 mL/min with UV detection was performed at 225 nm. The retention times of the enantiomers: (*S*)-**2** (17,32), (*R*)-**2** (18.60).

The enantiomeric ratio (*E*) was calculated in all cases using the equation reported by Chen et al.[18]

Acknowledgements

This work was supported by the Spanish CICYT (project BIO2001-2259). Authors thanks to Hispanagar SA for the gift of glyoxyl-agarose. We also thank Resindion Srl for donation of the amino-Sepabeads, Dr. Terreni for the kind supply of compound **2** and Angel Berenguer for interesting suggestions.

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