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On the kinetics of the enzyme-catalyzed hydrolysis of axial chiral alkyl allenecarboxylates: preparation of optically active *R*-(−)-2-ethyl-4-phenyl-2,3-hexadiene-carboxylic acid and its optically pure *S*-(+)-methylester

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Abstract

Pig liver esterase (PLE) was used for the preparation of optically active alkyl allenecarboxylates with axial chirality. Free and immobilized enzymes were used as biocatalysts for the kinetic resolution of racemic ester substrates. Whereas the biotransformations using the free biocatalyst resulted in moderately to high enantiomeric ratios, the immobilization significantly decreased the E-value. The reaction conditions were optimized with respect to the enantiomeric ratio and scaled up. The enantiomeric ratio (E-value) was thereby enhanced by a factor of four to $E = 60$. Under optimized conditions (free enzyme, addition of acetone as a cosolvent and Triton X-100 as an emulgator) in a preparative scale biotransformation, 282 mg of optically pure *S*-(+)-2-ethyl-4-phenyl-2,3-hexadiene-carboxylic acid methylester (96% ee, 82% yield) and 257 mg of *R*-(−)-2-ethyl-4-phenyl-2,3-hexadiene-carboxylic acid (83% ee, 80% yield) could be synthesized from the racemic substrate. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Allenes—whether chiral or not—played no role in preparative organic chemistry for a long time. However, during the last two decades not only a number of efficient and general methods to prepare cumulenic bond systems carrying all types of substituents have been developed but various routes to enantiopure or at least enantioenriched compounds have been described as well [1,2]. Chiral allenylsilanes—to mention but one example to illustrate this rapid progress in allene chemistry—have been employed as useful annulation reagents providing cyclopentenes with α,ß-unsaturated ketones and dihydrofuranes with aldehydes [3]. Beginning with the characterisation of mycomycin **1**, the first genuine natural allene [4] (Scheme 1), progress in the area of naturally occurring allenes has also been rapid although to date it is unclear whether

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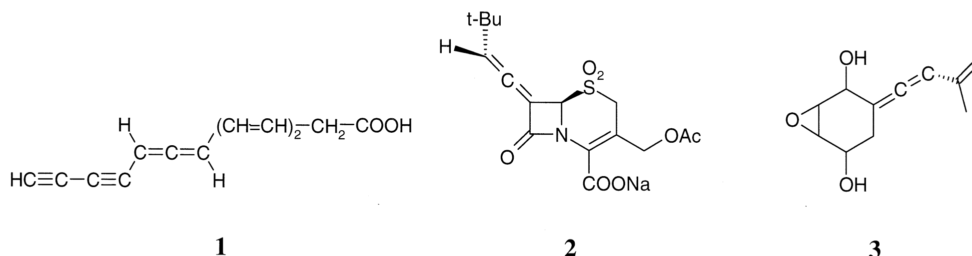


Scheme 1. Naturally occurring axial chiral allenes.

there are specific enzymes responsible for allene formation in nature. Of more recent interest are the biologically active allenes **2** and **3**: the former being a ß-lactamase inhibitor [5,6], and the latter a plant pathogen which has caused extensive damaging to vines [7].

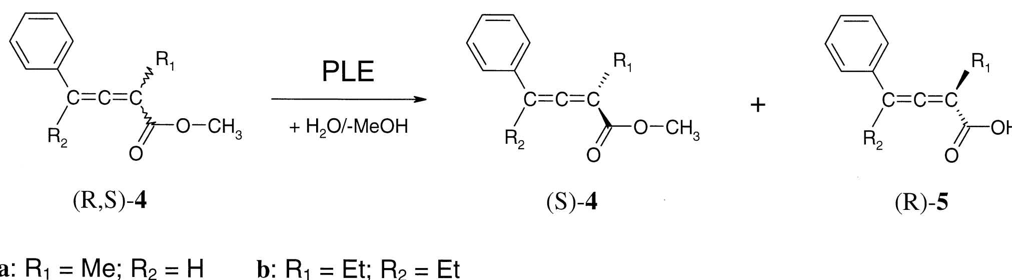
For the preparation of optically active allenes, especially of active allenecarboxylic acids, in which we are interested, practically all methods presently used in enantioselective synthesis have been employed. Thus, Ramaswamy et al. [8] have shown that pig liver esterase (PLE)-catalyzed hydrolysis of variously substituted allenic esters proceed with enantiomeric selectivity. However, as stated by the authors themselves, none of the reactions had been optimized. The results published so far cannot qualify as preparatively useful, i.e., providing amounts of optically active material large enough to allow further transformations. In fact, these earlier biotransformations were primarily carried out to gain an insight into the geometric restrictions of the active site of PLE rather than with an application on the laboratory scale in mind.

To develop such a method, we have therefore prepared two allene esters **4a** and **4b**, and subjected them to PLE catalyzed hydrolysis (Scheme 2). The results employing both the free and the immobilized biocatalyst, which is easier to handle as well as the optimization of the reaction conditions for the free enzyme are discussed.

2. Experimental

2.1. Chemicals and enzyme

Unless otherwise stated all chemicals used were of reagent grade and purchased from Fluka Chemie (Buchs, Switzerland). Pig liver esterase (PLE) was purchased from Sigma (Deisenhofen, Germany, Lot No. see below). The solution of salts and acids used in the procedures was prepared with deionised water.



Scheme 2. Reaction scheme of the pig liver esterase (PLE)-catalyzed kinetic resolution of alkyl allene carboxylates **4a** and **4b**.

*2.2. Preparation of R,S-2-methyl-4-phenyl-2,3-butadiene-carboxylic acid methylester (**4a**) and R,S-2-ethyl-4-phenyl-2,3-hexadiene-carboxylic acid methylester (**4b**)*

4a was synthesized according to the literature [9]. **4b** was synthesized by modifying the method described by Kresze et al. [10] for the ethylester. An α-bromo-butyric acid methyl ester (Aldrich, Steinheim, Germany) was used as a starting material.

2.3. Determination of the specific activity of the PLE: standard assay

The standard assay of the activity of the PLE was carried out as described by Horgan et al. [11] using butyric acid ethylester as a substrate. One unit (U) is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmol substrate/min.

*2.4. Bioconversion of allenes **4a** and **4b** using free and immobilized PLE*

2.4.1. Immobilization

The immobilization of PLE (Lot No: 32h8010, specific activity: 185 U/mg) to Eupergit C (Röhm Pharma, Darmstadt, Germany) was carried out according to Laumen et al. [12]. The specific activity of the immobilize was determined to be 128 U/mg bound protein (55% of the free enzyme, Laumen: 68%).

2.4.2. Bioconversion

Some 75 μmol of allene **4a** (14.1 mg) or **4b** (17.25 mg), respectively were added to 50 ml of 0.1 M phosphate buffer, pH 7.5 using a hamilton syringe. This mixture was then ultrasonicated for 20 min. The emulsion formed was temperated to 37°C and 1.560 mg of the free enzyme (290 U, PLE, Lot No.: 32H8010) or immobilized PLE (290 U), respectively were added.

Under stirring, the pH of the reaction was maintained at 7.5 by automatic titration of 0.1 M NaOH (Titrino 102, Methrom, Herisau, Switzerland). The conversion was calculated from the amount of base consumed during the reaction. Samples of 1 ml were extracted with 1 ml ethylacetate and analysed by gas chromatography.

*2.5. Optimization of the reaction conditions for the hydrolysis of **4b***

2.5.1. Substrate emulsions

(i) Some 8.1 mg (0.035 mmol) and (ii) 78.4 mg (0.272 mmol) allene **4b** were dissolved in each 500 μl of acetone and 500 μl of a solution of Triton X-100 in water (10 g/l) was added to stabilise the emulsion. A 2.5 ml of potassium phosphate buffer, (1.0 M, pH 7.4) and water was added to a final volume of 25 ml (final buffer concentration 0.1 M). This mixture was then ultrasonicated for 20 min to form a stable emulsion.

2.5.2. Bioconversion

The substrate emulsions were temperated to 37°C and the reactions were started by the addition of (i) 59 U and (ii) 450 U PLE (Lot No.: 115H7095, specific activity: 300 U/mg protein).

The pH of the reaction was maintained at 7.5 by automatic titration of 0.1 M NaOH (Titrino 102, Methrom, Herisau, Switzerland). The conversion was calculated from the amount of base consumed during the reaction.

Samples of 500 μl were extracted with the same amount of cyclohexane. The organic phase was dried over $MgSO_4$ and analysed by gas chromatography.

*2.6. Preparative bioconversion of allene **4b** by PLE*

2.6.1. Substrate emulsion

Some 345.5 mg (1.5 mmol) allene **4b** were dissolved in 20 ml of acetone. Then 20 ml of a

solution of Trition X-100 in water (10 g/l) were added and gently shaken. Five hundred milliliters of deionised water and 100 ml of 1.0 M potassium phosphate buffer, pH 7.4 were added and after shaking the volume was adjusted to 1000 ml with water. Sonication for 15 min yielded a stable substrate emulsion.

The procedure was repeated and the resulting emulsions were combined to a final volume of 2000 ml containing 3.0 mmol allene **4b**.

2.6.2. Bioconversion

In a 3 l vessel, the substrate emulsion was temperated to 37°C and titrated to pH 7.5 before 1109 μl enzyme suspension (5000 U, 16.64 mg PLE, Lot No.: 115H7095) were added.

The pH of the reaction was maintained at 7.5 by automatic titration of 0.1 M NaOH (Titrino 102, Methrom, Herisau, Switzerland). After 130 min, the reaction mixture was extracted twice with 600 ml portions of cyclohexane. The combined organic phases were washed three times with 200 ml saturated sodium chloride solution to remove the Triton X-100. After concentration to approximately 100 ml by rotary evaporation, the remaining allencarboxylic acid **5b** was removed by repeated extraction (two times) with 50 ml of 1.0 M $KHCO_3$-solution each. After drying over $MgSO_4$, the solvent was removed and 282 mg (82%) of allene ester **4b** was obtained.

The enantiomeric excess of the allene ester *S*-(+)-**4b** measured by gas chromatography was determined to be 96%. $[\alpha]_D^{25} = +66.1$ ($c =$ 0.096 g/100 ml, EtOH) corresponding to 96% ee. Lit: $[\alpha]_D^{25} = +53.9$ ($c = 1.74$ g/100 ml, EtOH) [13]. ^{1}H − NMR data and elementary analysis were identical with those of the substrate.

The water phase of the bioconversion was acidified to pH 2.5 by addition of 1.0 M phosphoric acid and 400 g of NaCl was added. Extraction with diethylether (three times with 300 ml each), drying over $MgSO_4$, and evaporation yielded 257 mg (80%) allene acid **5b**. $[\alpha]_D^{25} = -36.3$ ($c = 0.109$ g/100 ml, EtOH, 83% ee (chiral HPLC). Lit: $[\alpha]_D^{25} = -39.5$ ($c = 0.017$ g/100 ml, EtOH) [10]. ^{1}H-NMR data and elementary analysis were identical with those in the literature [10].

The isolated overall yield (**4b** and **5b**) was calculated to be 81%.

2.7. Analytical methods

2.7.1. Chiral GC

The separation of the enantiomers of allene methylesters **4a** and **4b** was accomplished using a octakis-2.6-(dimethyl-3-pentyl)-γ-cyclodextrine capillary column (9 m × 0.25 mm) [14]. Nitrogen was used as carrier gas at a pressure of 35 kPa. Temperature program: **4a**: 10 min, isotherm 80°C, 1°C/min to 96°C, 42 min isotherm 96°C: $\alpha = 1.04$; **4b**: 60 min, 102°C isotherm, $\alpha = 1.06$.

Using a heptakis-(6-*O*-tert-butyldimethylsilyl-2,3-di-*O*-methyl)-ß-cyclodextrine column (15 m × 0.25 mm) provided by Prof. A. König (Hamburg, Germany), the enantiomers of allene **4b** could be separated as well (Fisons Instruments GC [Rodano Milan, Italy] (carrier gas: H_2; pressure: 50 kPa; flow: 1.22 ml/min; split 1:100; temperature: 105°C isotherm; injection volume: 1.2 μl; detector and injector temperatures: 250°C): *R*-(−)-**4b**: 38.5 min; *S*-(+)-**4b:** 39.5 min; $\alpha = 1.03$.

2.7.2. Chiral HPLC

The separation of the enantiomers of **5b** was accomplished using a Spectra System (Thermo Separation Products) equipped with an ET 200/4 Nucleodex β-PM column (Macherey–Nagel). Solvent: 40 mM sodium phosphate buffer, pH 3.7/MeOH 60:40 (v/v), flow rate: 0.7 ml/min, pressure: 157 bar. **5** was detected at a wavelength of 241 nm. *R*-(−)-**5b**: 91 min; *S*-(+)-**5b:** 82 min; $\alpha = 1.11$.

Optical rotation was measured using a Perkin Elmer polarimeter (Model 341).

3. Results and discussion

3.1. Conversion of allenes **4a** and **4b** using free and immobilized PLE

Both substrates were emulgated in a buffered system and the reaction was started by addition of either the free or the immobilized enzyme. The enantiomeric excess was determined by gas chromatography as described in the experimental section, and the progress of the saponification was monitored by titrating the amount of acid formed during the reaction. In Fig. 1, the results for the two substrates are shown in graphical form.

The solid lines in the two graphs were calculated using the model of Chen et al. for computing the enantiomeric ratio ('*E*-value') [15]. Comparison of the results obtained with the free enzyme shows that allene **4b** is hydrolyzed with pronouncedly higher stereoselectivity than **4a** for any given degree of hydrolysis. Furthermore, the fit between experimental and computed curves is much better for **4b** than for **4a**. It is one of the conditions of the model mentioned above that the reaction of both enantiomers takes place at the same active site. It is, however, known that each PLE preparation consists out of several isoenzymes [16–19], which may be active at the same time, thus making it unlikely to fulfil this condition. The better fit for **4b** could hence indicate that only this substrate is converted by one of the isoenzymes.

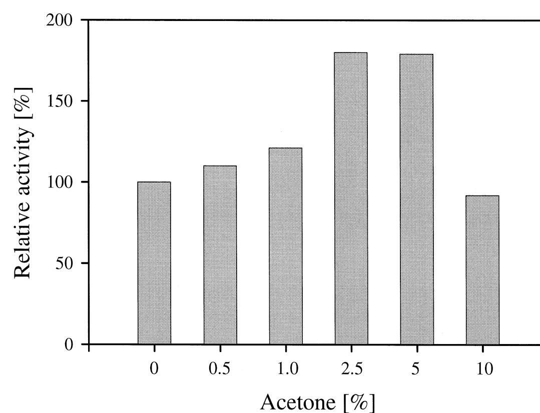


Fig. 2. Influence of the acetone concentration on the activity of PLE. Conversion of **4b**.

In comparison to the free enzyme the immobilized catalyst system shows a pronounced reduction in enantiomeric excess at any given time of the hydrolysis. Most likely this effect is caused by a change in the rate-determining step, with the diffusion of the substrate to the enzyme at the immobilization becoming rate-limiting under the latter conditions. This leads to a reduction of the hydrolysis rate of the better saponified *R*-enantiomer while the undesired hydrolysis of the *S*-enantiomer becomes more competitive.

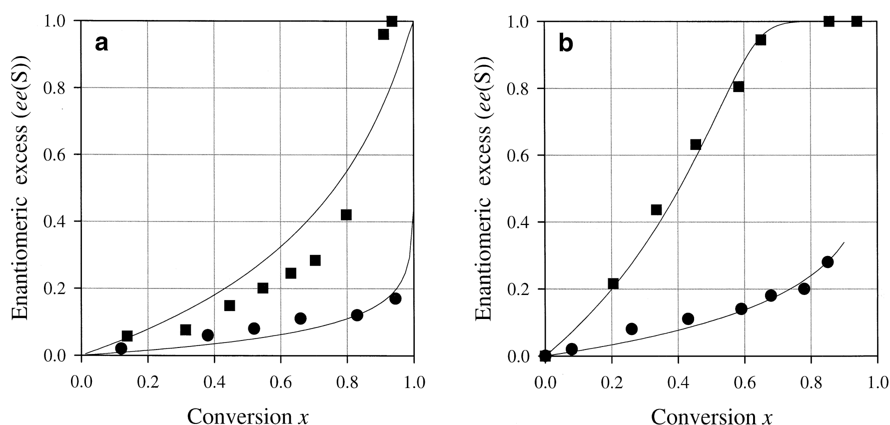


Fig. 1. Enantiomeric excess as a function of the conversion of **4a** (left hand graph) and **4b** (right hand graph). Free (■) and immobilized (●) pig liver esterase. Solid lines were calculated according to Chen et al. [15].

Whereas in the literature [8] 10% of **4a** was hydrolyzed (free enzyme) after 6 h to only 7% *R*-(−)-acid (16% ee) leaving 81% of the allene ester with almost no optical purity (1% ee), it can be seen from Fig. 1 that at a conversion of 95%, an ee > 99% can be obtained for **4a**. The optimization and the preparative scale bioconversion described below were carried out for **4b** because of the higher yield which could be expected from the higher enantiomeric ratio (E).

3.2. Optimization of the reaction conditions for the preparation of S-4b

In order to minimise the reaction volume for the intended preparative scale bioconversion of **4b**, the reaction conditions were optimized. Organic solvents are commonly used to enhance the solubility of substrates, and a positive effect on the enantiomeric ratio can often be observed too [20]. Therefore, the influence of different concentrations of acetone as a cosolvent on the activity of the PLE was investigated. As can be seen from Fig. 2, the addition of acetone resulted in a significantly enhanced activity of the pig liver esterase as measured under standard assay conditions.

Triton X-100 was added to stabilise the substrate emulsion. In concentrations up to 200 mg/l, Triton X-100 had no significant influence on the activity of the enzyme as measured under standard assay conditions (data not shown).

Following these basic experiments, the influence of acetone (2%, v/v) and Triton X-100 (200 mg/l) on the activity and the enantioselectivity of the PLE catalyzed hydrolysis of **4b** was investigated. In order to reduce the working volume for a planned preparative scale bioconversion, an additional experiment was carried out at a higher substrate concentration whereas the relation of enzyme activity to the amount of substrate was held constant at approximately 1650 U/mmol.

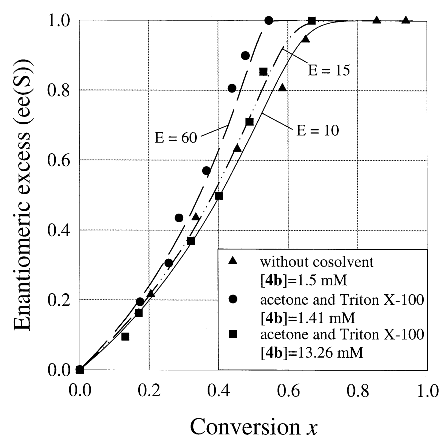


Fig. 3. Enantiomeric excess of the substrate as a function of the conversion. PLE-catalyzed hydrolysis of **4b** without and with cosolvents (free enzyme, 2% v/v acetone, 200 mg/l Triton X-100).

In Table 1, the initial reaction rates (activities) and the enantiomeric ratios (E) of all bioconversions discussed are summarised.

In the presence of acetone and Triton X-100, both the enantiomeric ratio E and the initial activity of the enzyme are significantly enhanced. However, the enhancement of the substrate concentration resulted in a reduced activity and a reduced enantiomeric ratio. Because the relation between enzyme and substrate as well as the concentration of the cosolvents was

Table 1
Summary of the results obtained during the optimization of the reaction conditions for the PLE-catalyzed kinetic resolution of **4a** and **4b**

Allene	Concentration [mM]	PLE	Acetone [%]	Triton X-100 [mg/l]	Activity [U/$mg_{protein}$]	E^a
4a	1.50	immobilized	0	0	0.16	1
	1.50	free	0	0	0.36	2
4b	1.50	immobilized	0	0	0.41	1
	1.50	free	0	0	2.29	10
	1.41	free	2	200	3.33	60
	13.26	free	2	200	1.15	15

[a]The enantiomeric ratio was calculated according to Chen et al. [15].

fixed this observation might be explained by an enhanced diffusion limitation of the substrate caused by an enhanced radius of the substrate drops. Although a higher activity seems to be correlated with a higher enantioselectivity in the case of added cosolvent, this is not seen in the absence of cosolvent.

Because of the high enantiomeric ratio of $E = 60$ in the case of 1.41 mM allene ester **4b** as a starting concentration (see Fig. 3 for the change in the enantiomeric ratio during the optimization), we linearly scaled-up these reaction conditions for the preparative synthesis of **4b**.

Whereas in the literature [8] **4b** was hydrolyzed up to 35% after 2 days (unknown amount of enzyme) yielding unknown amounts of the (*R*)-(−)-acid (93% ee, 17% yield) and of the (*S*)-(+)-ester (22% ee, 79% yield), the procedure described in the experimental section of this communication allowed the preparation of 257 mg (*R*)-(−)-acid (83% ee, 80% yield) and 282 mg of *S*-(+)-ester (96% ee, 82% yield) from 691 mg **4b** (overall yield 81%). Using 16.6 mg PLE, this biotransformation took 130 min.

Acknowledgements

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