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Lipase-Catalyzed Kinetic Resolution of a Series of Esters Having a Sulfoxide Group as the Stereogenic Centre

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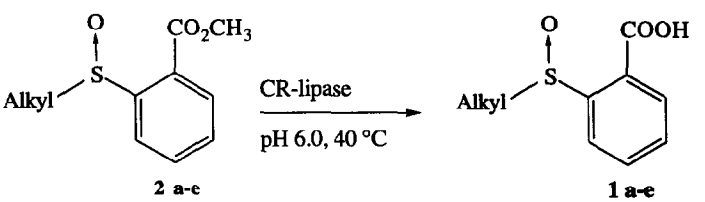
Abstract: A series of methyl 2-(alkylsulfinyl)benzoates (alkyl = C_1, *n*-C_4, *n*-C_8, *n*-C_{12} and *n*-C_{16}) was investigated with respect to substrate behaviour and enantioselectivity in a lipase (*Candida rugosa*)-catalyzed hydrolytic reaction. Although three bonds separate the stereogenic centre and the ester carbonyl group, very high enantioselectivity values (>100) could be obtained. It was found that the enzyme consistently showed a strong kinetic preference for the (-)-(S)-enantiomer.

Introduction

Lipase mediated hydrolytic reactions are of great synthetic interest since this group of enzymes accept a broad substrate variability, often coupled with a high enantioselectivity.[1] The microbial *Candida rugosa* and *Pseudomonas cepacia* lipases[2] are among the most interesting in this respect and have been successfully used as enantioselective catalysts for a variety of reactions involving esters.[3] The enantioselectivity obtained by the *Candida* enzyme under varying conditions has been carefully studied by Sih and collaborators,[4] who also showed that pretreatment of the enzyme[5] as well as addition of certain alkaloids, notably dextromethorphan,[6] to the reaction as allosteric inhibitors, could significantly improve the enantioselectivity.

The natural target molecules employed by lipases are fats and oils. However, long-chain containing substrates have not received much interest as study objects to better understand the lipase mechanism of action, although esters of methyl-branched octanoic acids as substrates have been reported to give a lipase activity strongly dependent upon the site of substitution.[7]

The use of chiral liquid chromatography as an analytical tool has greatly facilitated studies of enzyme-catalyzed reactions of this type[8] and further improved the precision of e.e. and enantioselectivity determinations. In this paper, as part of an investigation on structure-activity relationships, we present the enantioselective hydrolysis of a series of esters, possessing a sulfoxide group as the sole stereogenic centre located three bonds away from the ester carbonyl function (Scheme 1).



2 a-e **1 a-e**

Scheme 1. **a** = C_1, **b** = *n*-C_4, **c** = *n*-C_8, **d** = *n*-C_{12} and **e** = *n*-C_{16}

Results and Discussion

The retention behaviour of the racemic acids, **1a-e**, on the analytical liquid chromatography column used to determine the enantiomeric composition of the product from the enzymatic reaction, is given in Fig. 1. The elution order of **1a** was determined by injection of the respective enantiomers of known absolute configuration.[9] Fig. 1 shows that there is no reversal of the elution order on increasing chain length, since the separation factor α ($\alpha = k'_2/k'_1$) increases progressively in the series. The constant elution order, (+)-(R) prior to (-)-(S), was also verified by on-line polarimetric detection.

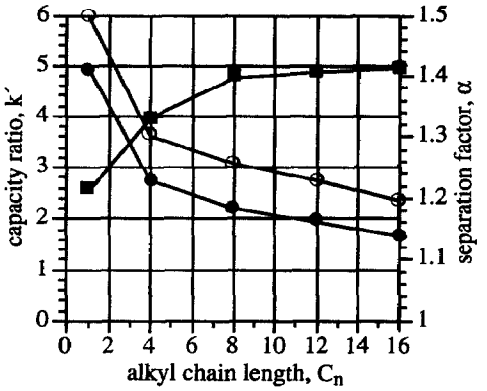


Fig. 1. Chromatographic retention behaviour of the series **1a-e**. Circles denote capacity ratios of the first (filled) and last (open) eluted enantiomers; squares denote α-values.

To investigate the effect of the alkyl chain-length on the overall rate of the enzymatic hydrolysis, competitive experiments with pairs of esters were performed, which had the advantage that identical reaction conditions was created, giving highly precise rate ratios. Tentative studies of the esters **2 a-e** separately, showed the hydrolysis rate of **2c** (alkyl = *n*-C_8) to be fastest, and consequently this substrate was used in competitive experiments with the others. The rate optimum for **2c** is clearly seen in Fig. 2. The ester **2a** (alkyl = C_1) did not hydrolyze at all.

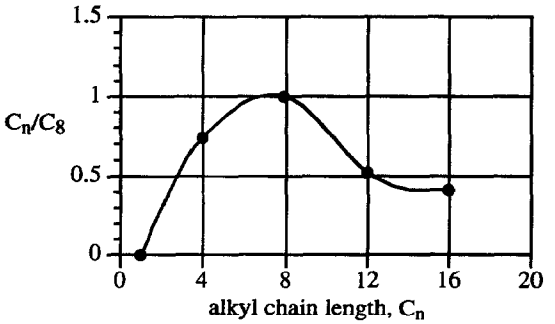


Fig. 2. Graph illustrating the chain-length dependence of the reaction rate. The C_n/C_8 value is the ratio between the (S)-enantiomers of the products formed after 3000 min.

The discrimination between the two enantiomeric substrates by the enzyme is expressed[10] by the enantioselectivity, *E*, according to eqn 1.

$$E = \frac{\ln[1 - c(1 + ee_p)]}{\ln[1 - c(1 - ee_p)]} \qquad (1)$$

The time-dependent parameters *c*, ee_p and ee_s are interrelated by eqn 2.

$$c = ee_s / (ee_s + ee_p) \qquad (2)$$

Eqn 1, which only holds for a completely irreversible reaction (kinetic resolution), is readily derived[4] from the rate ratio expressed by eqn 3, which is a time-independent measure of the selectivity shown by the enzyme.

$$E = \frac{(k_{cat}/K_m)_1}{(k_{cat}/K_m)_2} \qquad (3)$$

The chromatographic chiral phase system possessed a resolution power sufficient to separate the enantiomers of the ester substrates as well as the carboxylic acid products as shown by Fig. 3. Hence, in single substrate kinetic runs it is possible to obtain ee_s and ee_p simultaneously. Since this permits c to be calculated from eqn 2, the value thus obtained can be compared to the one determined experimentally from the product/substrate ratio found using a conventional (achiral) liquid chromatographic system.

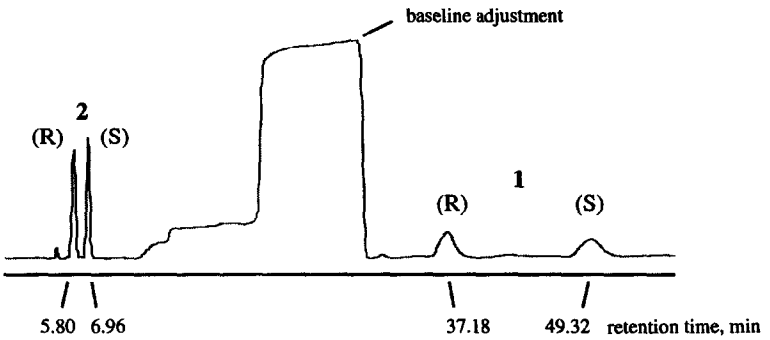


Fig. 3. A chromatogram illustrating the separation of the substrate and product enantiomers. An equimolar mixture of **1d** and **2d** was used. The artifact in the middle of the chromatogram is caused by the mobile phase change done immediately after elution of the ester enantiomers.

A characteristic feature of the lipases is their requirement of a phase boundary between an aqueous and an organic (substrate) phase in order to exhibit catalytic activity.[11] Since the substrates used were all solids, the reactions were run using octane as organic solvent. The octane/buffer phase system was efficiently mixed during the kinetic runs at constant temperature. Fig. 4 shows a result from a study of the hydrolysis of **2b**, which illustrates the high degree of discrimination between the two enantiomers obtained under these conditions. The octane content of the phase system was varied to investigate any possible influence on the initial reaction rate, but a variation of the content up to 50 % did not cause any significant effect.

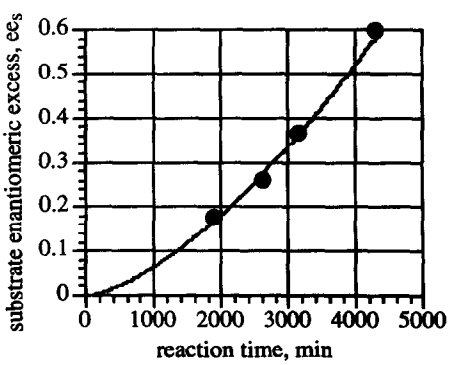


Fig. 4. The increase in ee_s as a function of time for the lipase-catalyzed hydrolysis of **2b**.

Since a precise determination of ee_s and ee_p is essential for the calculation of E, it was difficult to obtain reliable E-values, particularly at low values of c, from the competitive experiments. The two pairs of ester enantiomers were partly overlapping and the concentrations of the (R)-enantiomers of the acids formed were extremely low and hardly detectable. Therefore, a separate series of hydrolysis experiments with one substrate at a time was run. The chromatographic conditions were such that ee_s could be determined with high precision and the c-values could also be obtained from the same chromatogram after compensation for the slight difference in detector response between substrate and product. Table 1 summarizes the data obtained from these experiments. Very small changes in c produce large effects on high E-values, however, and therefore no exact figures are given for the substrates **2b** and **2d**. It seems likely that the chain length has a small, if any, influence on the enantioselectivity. The relatively small effect of the chain length (with the exception of C_1) upon the reaction rate suggests that the chain might be oriented into the organic phase, leaving the rest of the molecule accessible to the enzyme. In Fig. 5 the molecuar geometry of (-)-(S)-**2c** is shown.

Since the difference between the enantiomers lies in the orientation of the sulfinyl oxygen atom, it is reasonable to assume that the enzyme discriminates between the enantiomers due to a favourable hydrogen bond interaction with this oxygen atom in the most reactive enantiomer. Whether the effect is mainly caused by a change in K_M or in k_{cat} will be subject to further investigations.

Table 1. Conversion (c), substrate enantiomeric excess (ee_S) and enantioselectivity (E) values for the lipase-catalyzed hydrolysis of the reactive esters investigated.

Substrate	c	ee_s	E
2a	-	-	-
2b	0.255	0.365	>100[a]
2c	0.311	0.431	66
2d	0.229	0.306	>100[a]
2e	0.187	0.222	70

a) The errors in the determination of c are roughly of the order 1-1.5%. Therefore, it is not possible to specify E-values > ca 100.

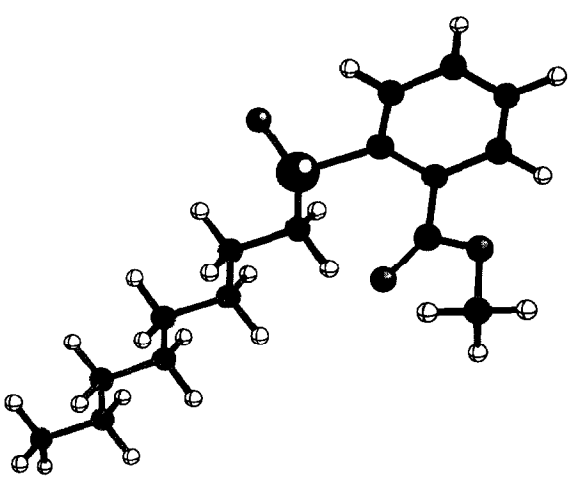


Fig. 5. Absolute configuration and preferred conformation of (-)-(S)-methyl 2-(octylsulfinyl) benzoate.

Experimental

Instrumentation. NMR spectra were obtained with a 400 MHz Varian VXR-400 spectrometer. The melting points were obtained by an Olympus BH-2 microscope with a Mettler FP 82 HT-Hot Stage heating cell interfaced with a Mettler Central Processor. The melting points are uncorrected. Analytical chiral liquid chromatography for determination of the enantiomeric composition was performed with the use of an equipment consisting of an LDC ConstaMetric mod. 3200 high-pressure pump, a Rheodyne injector with a 20 ml loop, the analytical column, and a ERC-7210 variable wavelength UV detector (Erma Optical Works) coupled to a Hewlett Packard mod. 3395 integrator. The column (4.6x200 mm), containing a Kromasil-based chiral sorbent,[12] was obtained from EKA Nobel AB, Bohus, Sweden. Analytical liquid chromatography (achiral) was performed with the use of an equipment consisting of an ERC mod. 64 high-pressure pump, a Rheodyne injector with a 20 µl loop, a Nucleosil 5µm C_8 or C_{18} column (4.6x150 mm), and a Perkin-Elmer mod. LC-15 UV detector coupled to Millipore/Waters model 740 integrator.

Chemicals. The thiosalicylic acid was from Sigma and the alkyl bromides were from Aldrich (Steinheim, Germany). The *Candida rugosa* lipase was from Sigma (prod. no. L1754). The enzyme was processed as described previously[5,8] in order to increase its enantioselectivity. Solvents for liquid chromatography were of HPLC- or spectroscopic grade from Fisons and Merck, respectively.

Synthesis. The compounds investigated were prepared from thiosalicylic acid as the starting material. The alkylation, carried out in an alkaline water/ethanol reaction medium, gave the 2-(alkylthio)benzoic acid in high yield. Oxidation to sulfoxide was performed with peracetic acid and the product composition determined by LC. The sulfoxides were recrystallized from acetic acid. The methyl esters were prepared by addition of an ether solution of diazomethane (generated from N-nitroso-N-methyl-*p*-toluenesulfonamide (Fluka) and distilled) to a solution of the sulfoxide in methanol, followed by evaporation of the solvent.

1a: ^{1}H-NMR (CD_3OD): δ 2.86 (s, 3H); 7.68 (ddd, 1H); 7.90 (ddd, 1H); 8.16 (dd, 1H); 8.19 (dd 1H)

2a: ^{1}H-NMR ($CDCl_3$): δ 2.86 (s, 3H); 3.96 (s, 3H); 7.58 (dd, 1H); 7.84 (dd, 1H); 8.09 (d, 1H); 8.32 (d, 1H)

1b: ^{1}H-NMR (acetone-d_6): δ 0.92 (dd, 3H); 1.46 (m, 2H); 1.59 (m, 1H); 1.86 (m, 1H); 2.60 (m, 1H); 3.16 (m, 1H); 7.69 (ddd, 1H); 7.91 (ddd, 1H); 8.18 (dd, 1H); 8.20 (dd, 1H)

2b: ^{1}H-NMR ($CDCl_3$): δ 0.95 (dd, 3H); 1.48 (m, 2H); 1.68 (m, 1H); 1.96 (m, 1H); 2.67 (m, 1H); 3.15 (m, 1H); 3.95 (s, 3H); 7.56 (ddd, 1H); 7.81 (ddd, 1H); 8.09 (dd, 1H); 8.26 (dd, 1H); Element. anal., (Found: C, 56.9; H, 6.1; S, 11.9; Calc. for $C_{12}H_{16}SO_3$: C, 60.0; H, 6.7; S, 13.3 %)

1c: ^{1}H-NMR ($CDCl_3$): δ 0.85 (t, 3H); 1.25 (m, 8H); 1.38 (m, 1H); 1.48 (m, 1H); 1.69 (m, 1H); 1.96 (m, 1H); 2.76 (m, 1H); 3.19 (m, 1H); 7.60 (ddd, 1H); 7.84 (ddd, 1H); 8.18 (dd, 1H); 8.28 (dd, 1H)

2c: ^{1}H-NMR (acetone-d_6): δ 0.88 (t, 3H); 1.29 (m, 8H); 1.45 (m, 2H); 1.63 (m, 1H); 1.89 (m, 1H); 2.58 (m, 1H); 3.14 (m, 1H); 3.94 (s, 3H); 7.68 (ddd, 1H); 7.91 (ddd, 1H); 8.13 (dd, 1H); 8.19 (dd, 1H); Element. anal., (Found: C, 60.5; H, 7.2; S, 11.5; Calc. for $C_{16}H_{24}SO_3$: C, 64.8; H, 8.2; S, 10.8 %); mp 47-50°C

1d: ^{1}H-NMR ($CDCl_3$): δ 0.87 (t, 3H); 1.22 (m, 16H); 1.38 (m, 1H); 1.47 (m, 1H); 1.69 (m, 1H); 1.96 (m, 1H); 2.76 (m, 1H); 3.18 (m, 1H); 7.60 (ddd, 1H); 7.84 (ddd, 1H); 8.18 (dd, 1H); 8.28 (dd, 1H)

2d: ^{1}H-NMR ($CDCl_3$): δ 0.88 (t, 3H); 1.25 (m, 16H); 1.43 (m, 2H); 1.71 (m, 1H); 1.96 (m, 1H); 2.67 (m, 1H); 3.14 (m, 1H); 3.95 (s, 3H); 7.56 (ddd, 1H); 7.81 (ddd, 1H); 8.09 (dd, 1H); 8.26 (dd, 1H); Element. anal., (Found: C, 66.9; H, 9.3; S, 9.2; Calc. for $C_{20}H_{32}SO_3$: C, 68.1; H, 9.2; S, 9.1 %); mp 61-64 °C

1e: ^{1}H-NMR ($CDCl_3$): δ 0.87 (t, 3H); 1.24 (m, 24H); 1.38 (m, 1H); 1.47 (m, 1H); 1.69 (m, 1H); 1.96 (m, 1H); 2.77 (m, 1H); 3.19 (m, 1H); 7.60 (ddd, 1H); 7.84 (ddd, 1H); 8.18 (dd, 1H); 8.29 (d, 1H)

2e: ^{1}H-NMR ($CDCl_3$): δ 0.88 (t, 3H); 1.25 (m, 24H); 1.40 (m, 1H); 1.46 (m, 1H); 1.71 (m, 1H); 1.97 (m, 1H); 2.66 (m, 1H); 3.14 (m, 1H); 3.95 (s, 3H); 7.56 (ddd, 1H); 7.81 (ddd, 1H); 8.09 (dd, 1H); 8.26 (dd, 1H); Element. anal., (Found: C, 69.9; H, 9.9; S, 7.7; Calc. for $C_{24}H_{40}SO_3$: C, 70.5; H, 9.9; S, 7.8 %); mp 72-74 °C

Enzymatic hydrolysis of the methyl 2-(alkylsulfinyl)benzoates (2a-e). A hydrolysis procedure previously described[8a] was used with some modifications. The reaction was carried out at 40°C in a biphasic system composed of 1000 µl phosphate buffer (100mM, pH 6.0), 100 µl *n*-octane and 100 µl of the *Candida*-lipase solution which showed a protein content of 2.6 mg/ml.[13] Samples from the competitive experiments

were taken at three different points of time, *viz.* after 3000, 4050 and 5510 minutes. Samples from the single experiments were taken at four different times, *viz.* after 1900, 2620, 3160 and 4300 min. The reaction mixture was quenched with 2M HCl, extracted with two portions of chloroform and the organic layer was centrifuged, evaporated and reconstituted with mobile phase. Samples without lipase solution serving as blank controls were prepared, worked-up similarly and *viz.* after 5510 minutes. At this pH the non-enzymatic hydrolysis was practically negligible.

Chiral liquid chromatography. The residue was dissolved in mobile phase and analyzed to determine the extent and composition of enantiomers formed. In the competitive experiments the enantiomeric composition was analyzed with a mobile phase composed of 98% hexane/2% 2-propanol + 0.05% (v/v) HCO_2H, eluted at 1.2 ml/min and detected at 228.5 nm. In the single experiments of substrates **2b-e**, the substrate and product enantiomers were eluted stepwise and the enantiomeric composition was analyzed (same flow and wavelength as above) as follows: for **2b** and **c** the mobile phase was composed of 99.5% hexane/0.5% 2-propanol and 99.5% hexane/0.5% 2-propanol + 0.05% (v/v) HCO_2H, for **2d** and **e** the enantiomeric composition was analyzed with mobile phase composed of 99.75% hexane/0.25% 2-propanol and 99.5% hexane/0.5% 2-propanol + 0.05% (v/v) HCO_2H.
Detector response factors were determined from the integral areas obtained by chromatography of equimolar mixtures of ester and its corresponding acid under the conditions described above for the respective compounds.

Molecular modelling. The 3D molecular graphics was carried out on a VAX 3100 computer using an AM1 semi-empirical program[14] for molecular geometry optimization.

Acknowledgements

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References and Notes

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10. Experimentally, two of the three parameters: *c* (overall degree of conversion), ee_s and ee_p (enantiomeric excess values of substrate and product, respectively) need to be determined to permit a calculation of the enantioselectivity, *E*. The subscripts 1 and 2 in eqn 3 denote the most and least reactive enantiomer (here (S)- and (R)-, respectively).
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