Stereoselective benzylic hydroxylation of alkylbenzenes and epoxidation of styrene derivatives catalyzed by the peroxygenase of *Agrocybe aegerita*

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Supporting information

Content

1 Commercially available chemicals used in this study

(In alphabetical order for each supplier): *tert*-butylbenzene, *m*CPBA (*m*-chloroperbenzoic acid) 2-indanol, indene, *trans*-β-methylstyrene, (*S*,*S*)-methylstyrene oxide ((2*S*,3*S*)-2-methyl-3-phenyloxirane), phenylacetaldehyde, 1-phenylethanol, 1-phenylpentanol, 2-phenyl-2-propanol, styrene oxide, 4-oxo-TEMPO (2,2,6,6-tetramethyl-4-oxo-piperidin-1-oxy), α-tetralone, β-tetralone, 1-tetralol (1,2,3,4-tetrahydro-1-naphthol) (Aldrich); acetonitrile (MeCN), $CHCl_3$, CH_2Cl_2, peracetic acid, styrene, (*R*)-(+)-1-phenylethanol, (*S*)-(-)-1-phenylethanol, 2-phenylethanol, *i*-PropOH (Merck); acetophenone, indan, (*S*)-(+)-1-indanol, ethylbenzene, pentylbenzene, (*S*)-(-)-1-phenylpropanol, propylbenzene, tetralin (1,2,3,4-terahydronaphthalene) (Fluka); L-(+)-ascorbic acid (Riedel de Haen); butylbenzene (Supelco); cumene, 1,2-dihydronaphthalene, 1,4-dihydronaphthalene, 1,4-epoxy-1,2-dihydronaphthalene, 1-hydroxyindan, α-methylstyrene, α-methylstyrene oxide (2-methyl-2-phenyloxirane), *cis*-β-methylstyrene, propiophenone, (*S*)-(+)-1-tetralol ((*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol) (TCI Europe). ^{18}O-labeled hydrogen peroxide was obtained from ICON Isotopes (Summit, NJ).

2 Preparations

2.1 Enzyme preparation

The peroxygenase of *Agrocybe aegerita* (*Aae*APO) was purified by several steps of ultrafiltration and ion exchange chromatography.[1] The final *Aae*APO preparation (main isoform II) had a specific activity of 120 U mg^{-1} related to the oxidation of veratryl alcohol;[2] one unit of *Aae*APO per mL (1 U mL^{-1}) refers to an enzyme concentration of 0.31 μM.

2.2 Enzymatic conversion and sample preparation

Enzymatic conversions were carried out in a total volume of 1.0 mL in 1.5-mL HPLC vials vigorously magnetically stirred at room temperature (RT). Reaction was started by addition of 20 μL of an appropriately diluted *Aae*APO preparation to the receiver. The receiver consisted of potassium phosphate buffer pH 7 (10 mM final concentration), substrate and hydrogen peroxide (both at 1.0 mM final concentration). To ensure substrate solubility, the reaction mixture contained 20% (vol/vol) acetonitrile (MeCN). After 10 min, the reaction was assumed to be completed and products immediately analyzed by HPLC. For GC/MS analysis, samples were slightly acidified (not in case of olefins due to instability of the expected epoxides) and intensively mixed with 200 μL of CH_2Cl_2 for one hour. Then the CH_2Cl_2 extracts were used for GC/MS analysis without further treatment.

2.3 *rac*-1-Indanol

Enantiomerically pure (*S*)-(-)-1-indanol was racemized after a modified method of Wuyts et al. using hydrochloric acid to an enantiomeric excess less than 5% *S*-(-)-1-indanol.[3]

2.4 Methylstyrene oxides

Racemic epoxides of α-methylstyrene, *cis*-β-methylstyrene and *trans*-β-methylstyrene were obtained from a conformationally conservative Prileshayew synthesis after Hibbert and Burt modified using the respective methylstyrene and 0.5 M peracetic acid in $CHCl_3$ at 0°C (kept overnight).[4] The organic phases were washed with alkali and water. Then aliquots were mixed with 20% by volume MeCN and $CHCl_3$ was evaporated in large part at RT. The residue was taken up in an aqueous mobile phase (resulting in app. 45% MeCN) and purified by HPLC to obtain a racemic standard sample. β-Methylstyrene oxide diastereomers were separated by achiral GC/MS and compared with an authentic standard of (*S*,*S*)-*trans*-β-methylstyrene oxide. The earlier eluting *cis*-isomer gave a 70 eV mass spectrum very similar to that of the authentic *trans*-isomer showing the same fragments in slightly varied intensities. α-Methylstyrene oxide was confirmed by achiral GC/MS in comparison to an authentic standard.

2.5 Indene oxide (1,2-epoxyindan), 1,2-epoxy-3,4-dihydronaphthalene and 2,3-epoxy-1,4-dihydronaphthalene

Indene oxide and dihydronaphthalene oxides were prepared in a similar manner as methylstyrene oxides from indene and the respective dihydronaphthalenes using *m*CPBA instead of peracetic acid in CH_2CL_2 and treated equally to receive racemic standard samples. MS^2 mass spectroscopy of the three epoxides in 50% (vo/vol) MeCN aqueous solution revealed quasi-molecular ions $[M-H]^+$ (133 and 147 m/z) and several adduct ions $[M\text{-}MeCN\text{-}H]^+$ (+41 m/z). The processing of the respective isolated MeCN adduct ions revealed the molecular ions $[M]^+$. Both $[M]^+$ and $[M-H]^+$ gave a common fragment series of mass losses of M -17 m/z, -27 m/z and -29 m/z presumably referring to the loss of hydroxyl, vinyl and formyl for both epoxides. 1,4-Dihydronaphthalene oxide was further analyzed by GC/MS and its mass spectrum was aligned with literature data.[5]

2.6 Chromatographic purification of epoxides

In order to acquire racemic standard samples of epoxides, a purification method was developed based on the analytical resolution of epoxides on the same column as mentioned below (section 3.4). Methodology is briefly exemplified for *cis*-β-methylstyrene oxide. Injections of up to 100 µL of the product mixture in aqueous phase (~45% MeCN) were accomplished. Chromatographic starting conditions were the same as for analytical resolution. One and a half column volume prior to *trans*-β-methystyrene oxide (impurity) elution a linear gradient was run to 70% MeCN from 10.4 to 11.0 min. Flow was reduced to 0.02 mL min^{-1} at 13.0 min to concentrate the desired *cis*-isomer eluting in a huge peak within app. 10 to 20 min depending on the injection volume. After elution of that peak the flow was set to 0.5 mL min^{-1} again and residual substances were removed from the column with 95% MeCN. Fractions whose 258 nm UV signal was greater than 200 mAU were collected. The combined fractions were re-chromatographed and found to be homogenous containing only *cis*-β-methylstyrene oxide but not the *trans*-diastereomer as illustrated in figure S1. Other epoxides were collected in a similar way.

3 Analytical methods

3.1 General remarks

Identification of reaction products was achieved by comparing them with authentic standards using chromatographic separation as well as UV/Vis and MS detection methods. Mass spectral analysis was assisted by automated NIST library search. To determine absolute configuration and enantiomeric excess (ee), racemic as well as immixtures of enantiomers of known ee were resolved by GC or HPLC and elution orders were compared or assigned to literature. To quantify the ee, peak areas of enantiomers served for calculation. Signals used in HPLC were specific UV-traces of 4 nm bandwidth whereas for mass spectrometry, the extracted trace of the most intensive substance related ion was evaluated. In the case of indene oxide no baseline separation was obtained in chiral separation by HPLC. Therefore peak height ratio of the sample was compared to that of a racemic mixture.

Determination of ^{18}O-incorporation was accomplished using molecular ion peaks obtained from achiral GC/MS with 70 eV EI and APCI-LC/MS. ^{18}O-incorporation is expressed by the ratio of intensities of the ^{18}O isotopic peak to the sum of ^{16}O and ^{18}O isotopic peaks.

Liquid and gas chromatography was performed on achiral and chiral phases coupled to mass spectrometry as described below.

3.2 Achiral GC/MS

Aliquots of 1 µL of the separated organic phase were injected into the GC. To clarify reaction products, gas chromatography/mass spectroscopy was performed on an Agilent 6890 model gas chromatograph equipped with a 5793 mass selective detector using a Zebron ZB 1701 (30 m × 0.25 mm ID × 0.25 µm film thickness, Phenomenex) capillary column at a flow rate of 1.5 mL min^{-1} helium. Injection was accomplished in split mode at 280°C and a temperature program was run from 60°C attended for 4 min and raised with 20°C min^{-1} to 280°C held for 5 min. 70 eV EI mass spectra were recorded from 40 to 300 m/z in 0.1 m/z step size at a rate of app. 1 Hz.

3.3 Chiral GC/MS

To resolve respective enantiomers, chiral gas chromatography / mass spectroscopy was performed on a Varian 3800 Chrompack model gas chromatograph using a beta-DEX 120 chiral analytical capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness, Supelco) possessing good selectivity for medium-size enantiomers. Carrier gas was helium at a constant flow rate of 1.5 ml min^{-1}. Injection was accomplished in the temperature-programmed vaporization (TPV) mode starting at 80°C to exclude the solvent in large part. Transfer of analytes to the column was accomplished with split ratios of 2 to 10 while heating the injector with 200°C min^{-1} to a point 20°C greater than the individual substance-dependent separation column temperature at which chiral resolution was taking place most efficiently. A column temperature program was run starting with an initial temperature of 80°C that was held during the transfer and increased at a rate of 50 °C min^{-1} up to the initial separation temperature followed by an increase of 0.2°C min^{-1} until 3 min after the enantiomers had eluted. Initial column separation temperatures for 1-phenylethanol, 1-phenylpropanol, 1-phenylbutanol, 1-tetralol and 1-indanol were 123°C, 132°C, 146°C, 136°C and 115°C respectively. For styrene oxide, *cis*- and *trans*-β-methylstyrene oxide these temperatures were 127°C, 110°C and 118°C, respectively.

The GC was connected to a Saturn 2000 ion trap mass spectrometer operating in 70 eV EI scanning mode. Mass spectra were recorded from 40 to 300 m/z with variable ionization time at a rate of app. 1 Hz.

3.4 Achiral HPLC and LC/MS

HPLC was performed on a 1200 Series Agilent liquid chromatograph. A UV-Vis diode array detector was connected recording absorption spectra in a range from 210 to 400 nm in 1 nm

step size. Routinely, a Gemini C6-Phenyl analytical column (150 × 2.1 mm × 3 µm, Phenomenex, 50°C) was used. A mixture of 10 mM phosphoric acid and MeCN was run at 0.35 mL min^{-1} starting from 20% MeCN that was held over 3 min followed by a linear ramp to 85% MeCN within 15 min and attended there for additional 3 min. To resolve indanol isomers a gradient from 1% to 3% MeCN in 0.01% HCOOH + NH_3, pH 3.5 was run within 80 min. In LC-MS resolution of 1-phenylethanol and acetophenone was achieved isocratically (68% MeCN in 0.01% HCOOH + NH_3, pH 3.5) at 0.5 mL min^{-1} flow rate on a RP column (Synergi Fusion-RP, 150 × 2.1 mm × 4 µm, Phenomenex, 50°C). Product mixtures of enzymatic and chemical epoxidation were resolved by HPLC using a Gemini-NX-C18 analytical column (150 × 2 mm × 3 µm, Phenomenex, 55°C). β-Methylstyrene oxide diastereomers were eluted with aqueous 0.01% HCOOH + NH_3, pH 7.0 starting at 17% MeCN at a flow rate of 0.5 mL min^{-1}. Traces of *trans*-β-methylstyrene oxide (that had been formed by impurities of the *trans*-isomer in the *cis*-β-methylstyrene preparation) eluted at 13.0 min and the *cis*-isomer at 14.3 min. More hydrophobic impurities were removed from the column by a gradient starting from 15 min up to 95% MeCN within 5 min that was held for further 3 min. Resolution of indene oxide and 1,2-dihydronaphthalene oxide was achieved in a similar way.

3.5 Chiral HPLC

Resolution of chiral epoxides was achieved using a Kromasil (*S,S*) column (Whelk-O 5/100 250 × 4.6 mm × 5 µm, Regis Technologies Inc. IL, 40°C) that operated in the reversed phase mode and run isocratically with aqueous 0.01% HCOOH + NH_3, pH 7.0 containing a certain fraction of *i*-PropOH at a flow rate of 1.7 mL min^{-1}. (indene oxides: 10.0/11.2 min (30% *i*-PropOH), 1,2-dihydronaphthalene oxides: 12,8/13,1 min (33% *i*-PropOH), *cis*-β-methylstyrene oxides: 18.2/19.1 min, *S,S*-β-methylstyrene oxide: 29.7 min (25% *i*-PropOH)). In the normal phase mode, *cis*-β-methylstyrene oxides eluted in the same order: 3.72/3.78 min (*n*-hexane/*i*-PropOH 70/30, 1.0 mL min^{-1}). Absolute configuration of indene oxides was ascertained by the elution order according to literature.[6] To confirm absolute configuration of *cis*-β-methylstyrene oxides, the elution order after Tse et al.[7] on a Chiralpak IB column (250 × 4.6 mm × 5 µm Daicel, Ciral Technologies Europe, France, 40°C) was evaluated. Enantiomers eluted in opposite order on this column: (1*R*,2*S*)-/(1*S*,2*R*)-methylstyrene oxide 7.9/8.6 min (*n*-hexane/*i*-PropOH 70/30, 0.8 mL min^{-1}).

3.6 Determination of absolute conformation of (1*R*,2*S*)-methylstyrene oxide

The enzymatically formed epoxide enantiomer eluted earlier than the other one on a Chiralpak IB column indicating the opposite (1*R*,2*S*)-methylstyrene oxide ((2*S*,3*R*)-2-methyl-3-phenyloxirane) reported by Tse et al.[7] for a Chiralcel OD-H column possessing the same chiral descriptor. On a Kromasil (S,S) Whelk-O phase, the enzymatic product was the later eluting one both in the normal and in the reversed phased mode.

3.7 LC-MS of ^{18}O-products

LC-MS was performed to confirm/supplement the GC/MS data on ^{18}O-incorporation into acetophenone. An Agilent G1956A MSD-VL linear quadrupol mass spectrometer was used equipped with an atmospheric pressure chemical ionization (APCI) source that operated in positive ionization mode (4 kV, 10 nA emission current at 350°C). Achiral HPLC was run as described above.

3.8 MS^2

MS^2 was performed on an Agilent 6300 ion trap mass spectrometer (Bruker Daltonics) equipped with a switchable Agilent ESI/APCI (multimode ion source) interface. Sample application was achieved using a syringe pump (KDS 100, KD Scientific Inc. MA) at a flow rate of 20 µL min^{-1}. Ionization was accomplished in the positive APCI mode at 300°C.

3.9 Determination of kinetic parameters

Determination of kinetic parameters for C_α-hydroxylation is exemplified for ethylbenzene and propylbenzene. Due to the high similarity of the UV spectra of the substrate and the corresponding hydroxylated product, which obviates the direct photometric observation, a method on the basis of HPLC analysis was developed. Enzymatic conversions were carried out in triplicates in a total volume of 200 µL at RT (25°C) and the reaction was stopped by adding 20 µL of 50% (vol/vol) trifluoroacetic acid (TFA) after 15 s. To ensure convergence of the kinetic constants, three different enzyme concentrations (4.2, 8.4, 16.8 nM) were tested. The reaction mixture contained 20% (vol/vol) MeCN in 10 mM potassium phosphate (pH 7) to provide for the solubility of the substrate. Hydrogen peroxide concentration was held at 2 mM for all reactions. Values were processed by classical linearization methods (Lineweaver-Burk, Eadie-Hofstee, Hanes) as well as by nonlinear regression.[8]

4 Optimized production of (*R*)-1-phenylethanol

In order to optimize the production of (*R*)-1-phenylethanol, a fed-batch reaction design was developed at 1-mL scale with a receiver of 0.5 mL containing the enzyme in 10 mM

potassium phosphate (pH 7) buffered 20% (vol/vol) MeCN solution. To this mixture, 0.5 mL of a fed solution consisting of ethylbenzene and hydrogen peroxide in varying molar ratios (giving 20 mM in sum) in 50% (vol/vol) MeCN was pumped through a stainless steel capillary (0.17 mm ID) connected to a syringe pump (KDS 100, KD Scientific Inc. MA). By using this design, the parameters *Aae*APO concentration, feeding flow rate and molar ratio (H_2O_2/ethylbenzene) could be varied independently. A typical experiment took 30 min (at a flow rate 1 mL h^{-1}) and substrate/products were analyzed by HPLC.

5 References

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Table S1 Results of the enzymatic conversion of benzylic substrates by *Aae*APO. Reactions were carried out using 0.5 U mL^{-1} *Aae*APO, 1 mM of the substrate/hydrogen peroxide couple in 10 mM potassium phosphate buffer (pH 7.0) containing 20% MeCN (n.d. not determined).

Substrate	conversion	main product (ee)	further products
***n*-alkylbenzenes**			
ethylbenzene	95%	(*R*)-phenylethanol (>99%)	acetophenone in traces (52 µM)
propylbenzene	64%	(*R*)-(+)-1-phenylpropanol (>99%)	propiophenone in traces (45 µM)
butylbenzene	52%	(*R*)-(+)-1-phenylbutanol (~40%)	butyrophenone in traces (37 µM)
pentylbenzene	8.4%	1-phenylpentanol (n.d.)	valerophenone in traces (24 µM) three further minor products
styrenes			
styrene	71%	styrene oxide (7%)	
α -methylstyrene	96%	α -methylstyrene oxide (29%)	α-methyl phenylacetaldehyde by rearrangement
trans-*β*-methylstyrene	19%	3-phenyl-propen-1-ol 3-phenyl-propen-1-one	(*S,S*)-β-methylstyrene oxide (>98% ee)
cis-*β*-methylstyrene	95%	(1*R*,2*S*)- *cis*-β-methylstyrene oxide (>99%)	2 further products in traces
cycloalkyl benzenes			
tetralin (1,2,3,4-tetrahydronaphthalene)	85%	(*R*)-(-)-1-tetralol (>99%), 1-tetralone	146 m/z (very probably 2-tetralol), 2-tetralone in traces
1,2-dihydronaphthalene	95%	3,4-dihydronaphthtalene-1,2-oxide (32%)	naphthalene (<5%) and its conversion products[9]
1,4-dihydronaphthalene	~100%	1,4-dihydronaphthtalene-2,3-oxide[5]	3,4-dihydronaphthalen-2(1H)-one
indane	77%	*R*-(-)-1-indanol (>87%)	2-indanol, 1-/2-indanone in traces
indene	96%	indene oxide (2.3%)	*trans*-/*cis*-1,2-indane diol in traces

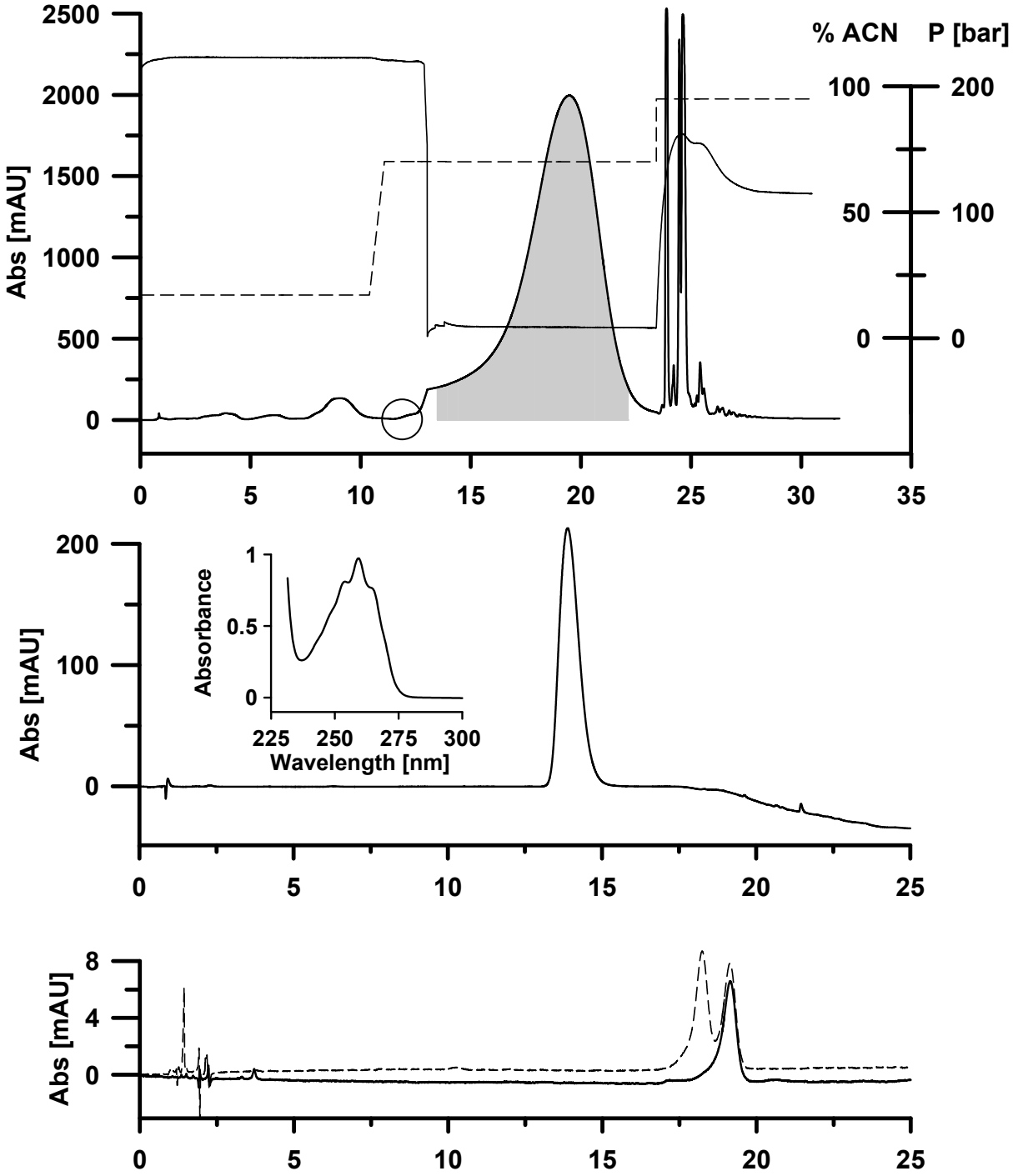

Fig S1 Chromatograms of the purification of *cis*-β-methylstyrene oxide and chiral resolution of the enantiomers. Top: separation of the *trans*- (small peak indicated by the circle at 12.4 min) from the *cis*-diastereomer (starting from 12.6 min) and the fraction collected indicated grey. Chromatographic parameters pressure (thin solid line) and organic portion (thin dashed line) are shown as explicated in the text. Middle: the collected fraction was re-chromatographed to show purity, Insert: UV spectrum of *cis*-β-methylstyrene oxide. Bottom: Chiral separation of the *cis*-β-methylstyrene oxide enantiomers, racemic fraction (dashed line) and the fraction obtained from enzymatic conversion of *cis*-β-methylstyrene.