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Epoxidation of Styrene and Substituted Styrenes by Whole Cells of *Mycobacterium* sp. M156

Stuart R. Rigby,[1] Colette S. Matthews[2] and David J. Leak[2]*

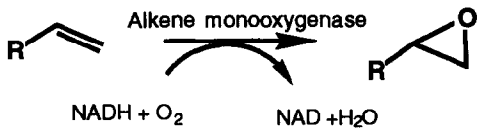
[1]*ZENECA Bio Products, P.O. Box 2, Belasis Avenue, Billingham, Cleveland, TS23 1YN, U.K.*

[2]*Centre for Biotechnology, Department of Biochemistry, Imperial College of Science Technology and Medicine, London SW7 2AZ, U.K.*

Abstract—Whole cells of the propene utilizing *Mycobacterium* sp. M156 (NCIMB 40156) oxidised styrene, 2-,3- and 4-fluorostyrene, 3- and 4-chlorostyrene and 3- and 4-methylstyrenes to their respective epoxides. Rates of oxidation were comparable to that of styrene. α-Methylstyrene was also epoxidised at a lower rate, while *trans*-β-methylstyrene and 1,2-dihydronaphthalene were poor substrates. In those cases that were investigated, epoxidation occurred with a high degree of stereospecificity.

Introduction

Bacteria isolated for their ability to grow on low molecular weight terminal alkenes (ethene, propene, 1-butene and butadiene) fall into a limited range of genera.[1] These are mainly comprised of *Mycobacterium* spp., *Nocardia* spp. and *Xanthobacter* spp. Most of these bacteria, particularly if they have been counter-screened against growth on the saturated alkane homologue to eliminate organisms potentially growing via ω- or (ω-1) hydroxylation, metabolize the alkene via terminal epoxidation using an alkene specific monooxygenase (Scheme I). As with many monooxygenases this enzyme appears to have a wide substrate tolerance,[1,2] although problems of enzyme stability and low recovered activities upon isolation have precluded a detailed study of purified or even crude preparations of isolated (cell free) enzyme. However, whole cells have been shown to epoxidise a range of non-polar terminal and sub-terminal alkenes including allyl-aryl ethers, yielding the (ω-1)(*S*)-epoxides[3,4] which have value as precursors for the synthesis of β-blockers, in high enantiomeric excess.



Scheme I.

We have been studying the enzymology and biocatalytic range of a *Mycobacterium* sp. M156, a propene oxidising bacterium which does not grow on alkanes, and provisionally identified as a strain of *Mycobacterium obuense*. We report here some studies of whole cell epoxidation of styrene and styrene derivatives.

Results

Biotransformation optimization

Mycobacterium sp. M156 grows on propene and butene and is unable to grow on higher alkenes because of the substrate specificity of the enzyme catalysing epoxide degradation. Hence, with more complex biotransformation substrates, such as styrene, the epoxide accumulates in the medium and is not further metabolised. Preliminary optimization studies showed that pH 7.5 and 37 °C were the optimum conditions for resting cell biotransformations of styrene (Figure 1a,b), while the optimum substrate concentration was 0.1 % (v/v). Substrate toxicity was evident at 0.2 % (v/v) (Figure 1b). Because of the small scale of the assay it was desirable to add the substrate in a larger volume of a water-miscible solvent. Acetone was well tolerated at 1 % (v/v) and was not cooxidised. However, methanol, ethanol and acetonitrile were all inhibitory at the same concentration. Hence, all substrates were added as 10 % (v/v) solutions in acetone, throughout.

All monooxygenases require a source of reducing equivalents, often in the form of NAD(P)H, for oxygen activation. In growing cells this is provided by the further metabolism of the substrate. In a resting cell biotransformation where the epoxide is accumulating, the reaction is dependent on the degradation of endogenous energy reserves. With M156 this can lead to significant levels of epoxide accumulation. However, when relying on endogenous reserves the initial rate of epoxidation was low, increasing to a maximum value (33 nmol min^{-1} mg dry $weight^{-1}$ for styrene oxide production) during the first 10 min of the assay. Therefore, to measure a meaningful initial rate of epoxidation, 5 mM sodium gluconate, a rapidly metabolisable substrate, was added to the cell suspension. The maximum rate, with or without gluconate, was very similar, suggesting that gluconate metabolism was not the rate determining step in the assay. However, the presence of gluconate ensured that the initial rate was also the maximum epoxidation rate and that this rate was maintained for up to 1 h. In the absence of gluconate the rate started to decline after 30 min of incubation.

As a result of these preliminary studies the optimized assay conditions comprised: 3.75 mg (dry weight) cells in 1 mL

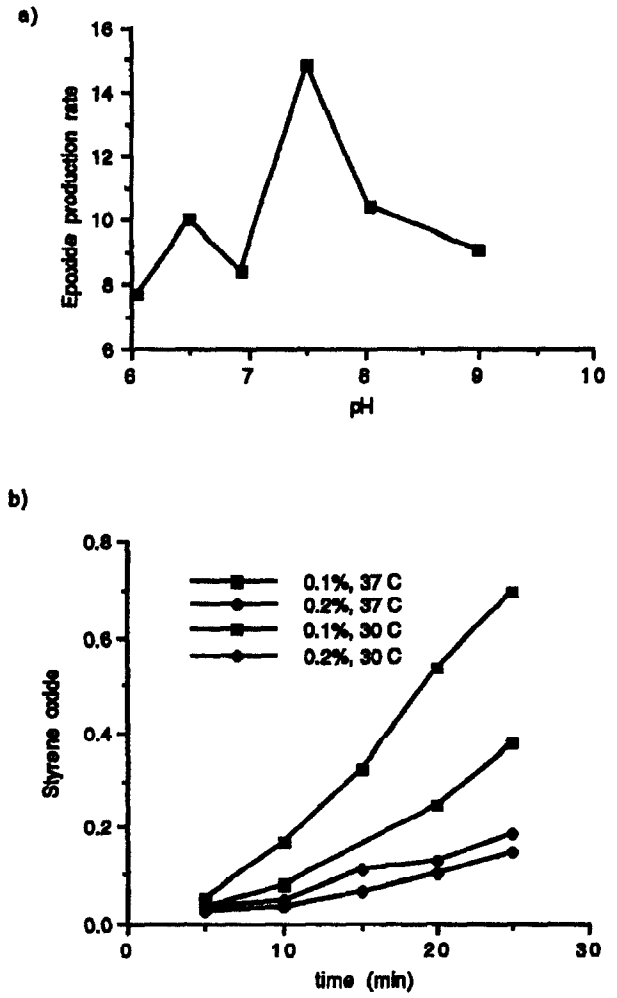


Figure 1. (a) Effect of pH on styrene oxide production rate (nmol min^{-1} mg dry $weight^{-1}$) using whole cells of *Mycobacterium* sp. M156 (30 °C, 0.1 % styrene); (b) Effect of temperature and substrate concentration (0.1 or 0.2 %) on the time course of styrene epoxidation (μmol mg dry $weight^{-1}$) by whole cells of *Mycobacterium* sp. M156.

25 mM phosphate buffer pH 7.5 containing 5 mM sodium gluconate, 37 °C, 0.1 % (v/v) substrate added as a 10 % (v/v) solution in acetone.

Substrate range

Using the optimized assay conditions the rates of epoxidation of some commercially available styrene derivatives were determined (Table 1). It should be noted that the rate of whole cell styrene epoxidation was similar to that observed with propene (the propene assay is routinely done at pH 9.0 which minimises further metabolism of epoxypropane, but is not the optimum pH for production of epoxides from non-growth substrates) suggesting that styrene is a good substrate for the enzyme and that there are no significant permeability barriers for styrene.

Ring substitution in the 3- and 4-positions reduced epoxidation rates slightly, although with whole cells it is difficult to distinguish between reduction in enzyme catalytic rate and decreased cell permeability. However, it is unlikely that permeability would vary in a series of regioisomers. Therefore, the lack of epoxidation of 2-chlorostyrene must reflect steric hindrance which is not evident with the less bulky fluoro substituent.

Table 1. Relative rates of epoxidation of various styrene derivatives by whole cells of *Mycobacterium* sp. M156. The rate of epoxidation of styrene under the optimised conditions was 33 nmol min^{-1} mg dry $weight^{-1}$

Substrate	Relative epoxidation rate
Styrene	100 %
2-Fluorostyrene	77 %
3-Fluorostyrene	92 %
4-Fluorostyrene	80 %
2-Chlorostyrene	-
3-Chlorostyrene	72 %
4-Chlorostyrene	68 %
2-Methylstyrene	nd
3-Methylstyrene	94 %
4-Methylstyrene	90 %
α-Methylstyrene	49 %
β-Methylstyrene	< 2 %
2-Methyl-1-phenylpropene	-
1,2-Dihydronaphthalene	< 2 %

(nd = not determined; - = no product detected)

Substituents closer to the site of epoxidation had a dramatic effect on epoxidation rate. The effects of α- and β-methyl substituents are consistent with the reduction in rate seen in comparisons of terminal alkenes with 2-substituted and subterminal aliphatic alkenes, although epoxidation of subterminal aliphatic alkenes usually occurs at about 30 % of the rate seen with the terminal homologue. The lack of epoxidation of 2-methyl-1-phenylpropene completes the pattern. We have previously observed that internal double bonds such as that present in 3-hexene are not epoxidised by M156 although simple cyclic alkenes e.g. cyclohexene are epoxidised slowly (unpublished results). The slow rate of epoxidation of 1,2-dihydronaphthalene is consistent with this.

Stereospecificity and product identification

Selected epoxidations were scaled up to provide material for chiral GC analysis, GC-MS and optical rotation measurements (Table 2). Enantiomers (ω-1)(*R*) and (*S*) of styrene oxide were used as standards to confirm that the (+)(*R*)-enantiomer was the major product of the biotransformation and this was supported by optical rotation measurements. However, significant quantities of phenylacetaldehyde and acetophenone were also detected by GC-MS. More detailed investigation revealed that the aldehyde was primarily arising as the result of thermal degradation in the GC injector. It is not clear at present, whether the ketone is a genuine biotransformation product or results from subsequent chemical isomerisation of the epoxide. Aldehyde and ketone peaks were also detected in the other biotransformations; with α-methylstyrene a major broad aldehyde peak suggested that thermal rearrangement was occurring on the column, indicating that chiral HPLC would probably be a better method of analysis.

Enantiomeric excess was high for all of the biotransformations. Although it is not possible to identify the major isomer from the optical rotations alone it is pertinent to note that with all of the ring substituted styrene oxides the major enantiomer was the first to elute

Table 2. Products formed in the scaled-up biotransformations of selected styrene derivatives. The enantiomeric excess (*e.e.*) of the epoxides formed was determined from the peak areas of the two enantiomers separated by GC on Lipodex C. α_D^{25} values were measured using the maximum concentrations available.

Substrate	Major product (% of total)	Minor products (% of total)	epoxide *e.e.*	α_D^{25}(+/-)
Styrene	R-epoxide (84.9)	S-epoxide(2.8) aldehyde (3.0) ketone (9.3)	93 %	+
2-fluorostyrene	epoxide (94.3)	epoxide (1.4) ketone (4.3)	97 %	-
4-fluorostyrene	epoxide (95.2)	epoxide (1.9) aldehyde (1.9) ketone (1.0)	96 %	nd
4-chlorostyrene	epoxide (92.3)	epoxide (3.2) ketone (4.4)	93 %	-
4-methylstyrene	epoxide	epoxide unidentified	>90 %*1	+
α-methylstyrene	aldehyde (rearranged)	epoxide	>90 %*2	nd

(nd = not determined; * = unable to determine accurately due to poor resolution of minor enantiomer from unknown product (*1) or low epoxide concentration (*2))

from the Lipodex C column. This, together with the knowledge that all epoxidations done with this organism have been stereochemically consistent (where the enantiomers could be identified) suggests that the (*R*)-isomer is likely to be the major product in each case.

Discussion

The alkene specific monooxygenases present in some bacteria isolated on low molecular weight alkenes have been shown to catalyse the epoxidation of simple terminal and subterminal alkenes with high stereospecificity.[1] The present study shows that, with *Mycobacterium* sp. M156, substituted styrenes are also epoxidised with a high degree of stereospecificity and at rates comparable to that of the natural substrate, propene. However, problems of substrate toxicity mean that any attempt to produce these epoxides on a larger scale would require a second, non-toxic organic phase to sequester the substrate and products. Nikko Kyodo Co. Ltd currently use such a two phase whole cell process for the commercial production of chiral epoxides using *Nocardia corallina* B-276.[5] However, as their organism grows on alkanes as well as alkenes it is unclear which monooxygenase[6] is catalysing the reaction. Certainly, styrene oxide produced by *N. corallina* B-276 is of much lower *e.e.* than that reported here.[5]

Where stereospecificity is observed, most alkane and alkene monooxygenases preferentially produce the (*R*)-epoxyalkane, although a *Pseudomonas* sp. isolated on styrene as sole carbon source[7] and containing an FAD dependent monooxygenase has recently been shown to produce (*S*)-styrene oxide.[8] However, unlike the alkane monooxygenases, no C-H bond oxidation is seen with the alkene specific enzymes. Hence, the stoichiometric conversion of alkenes to epoxides at high *e.e.* is a realistic prospect with organisms possessing this type of enzyme. The formation of small amounts of acetophenones in these biotransformations needs to be investigated further to establish whether they result from the initial monooxygenase reaction or subsequent rearrangement. We have previously observed ketone formation with internal alkenes, such as 3-hexene, as substrates (unpublished results). However, this has been attributed to poor substrate binding in the active site and is accompanied by very low conversion rates. In the present study, conversion rates were relatively high, suggesting that substrate binding was not a problem. Nevertheless, some chemical rearrangement of epoxides is likely to occur over longer incubation periods, such as might be used in larger scale production, due to the sensitivity of epoxides to acid catalysed hydrolysis and nucleophilic attack. A second (organic) phase would, therefore, be beneficial for sequestering the product as well as the substrate.

We are interested in understanding the differences between alkane and alkene monooxygenases which give rise to their different specificities. Current evidence suggests that, like the alkane monooxygenases such as methane monooxygenase, *w*-hydroxylase or the cytochrome P-450 class of enzyme,[9] the alkene specific enzymes are multicomponent and contain iron at the active site (unpublished results). In the alkane enzymes an iron-oxo intermediate is formed which is highly electrophilic. However, epoxidation could occur via electrophilic or nucleophilic attack and model iron-peroxo species have been shown to epoxidise olefins via nucleophilic attack.[10] Assuming that there are no differences in permeability, comparison of the rates of epoxidation of 2-, 3- and 4-fluorostyrenes suggests that the active oxygen species involved in the propene monooxygenase from M156 is also electrophilic, the electron withdrawing effects of the 2- and 4-fluoro substituents reducing the rate of epoxidation compared to 3-fluorostyrene.

Experimental

Chemicals

All of the biotransformation substrates were obtained from Aldrich Chemical Co. Ltd, Gillingham, Dorset, U.K. All gases were obtained from BOC, Crawley, U.K.

Organism

Mycobacterium sp M156 (NCIMB 40156) was isolated from soil samples using propene (10 % v/v) as sole carbon source and a nitrate mineral salts medium.

Cells were cultured at 34 °C in a 20 L Chemap fermentor with a 16 L working volume in a medium containing, per litre: NH_4Cl, 3 g; $MgSO_4.7H_2O$, 10 g; Na_2HPO_4, 3.3 g; KH_2PO_4, 2.6 g; $CaCl_2.2H_2O$, 500 mg; FeNaEDTA, 3.8 mg; 10 mL trace elements solution. Trace elements solution contained (per litre): $NiCl_2.6H_2O$, 10 mg; $MnCl_2.4H_2O$, 20 mg; $CuSO_4.5H_2O$, 500 mg; $CoCl_2.6H_2O$, 50 mg; $FeSO_4.7H_2O$, 500 mg; $ZnSO_4$, 2g; H_3BO_3, 10 mg; $NaMoO_4.2H_2O$, 500 mg. Propene (150–200 mL min^{-1}) was supplied as the carbon source as a gas mixture with air (900–1000 mL min^{-1}) and CO_2 (50 mL min^{-1}) which were pre-mixed in a gas blender (Signal Series 850). The agitation speed was initially 500 rpm increasing to 700/800 rpm during the fermentation to maintain the dissolved oxygen tension at around 20 % of saturation. The inoculum was 500 mL of a culture grown in the same medium and cells were harvested after 4–5 days at a cell density of 2.5–3g L^{-1}. Cells were initially pre-concentrated from 16 L to approximately 2 L by tangential flow microfiltration across a polysulphone hollow fibre filter (1 m^2; nominal molecular weight cut-off, 10^6) using a Bio-2000 Model 2 pump (Bio-Flo Ltd, Glasgow, UK) and subsequently centrifuged at 10,000 *g*, washed in 50 mM potassium phosphate buffer pH 7.3 and resuspended in a minimum volume of the same buffer. The resulting cell paste was 'drop frozen' in liquid nitrogen and stored at –70 °C until required.

Biotransformations

Initial optimization and rate determinations were carried out in 7 mL conical flasks containing 1 mL of 25 mM buffer and 3.75 mg cells. The flasks were sealed with a suba-seal (W.H. Freeman Ltd, Barnsley) and the cell suspension pre-incubated at 30 °C or 37 °C for 2 min before addition of the substrate. At the end of the incubation the flasks were plunged into ice and, when cool, the contents were extracted into ether. 1,2 Epoxy-3-phenoxypropane was included as an internal standard.

For larger scale biotransformations, 60 mL of cell suspension containing 2.5 mg mL^{-1} of cells was used under the optimised conditions (25 mM phosphate buffer pH 7.5, 37 °C, 5 mM gluconate, 0.6 mL of a 10 % (v/v) solution of the substrate in acetone, 60 min incubation). At the end of the incubation the cell suspension was cooled as before, the cells removed by centrifugation (10,000 *g*, 15 min) and the products extracted by solid phase extraction using 500 mg of Isolute C8 (EC) (IST Ltd, Hengoed, Glamorgan, U.K.) with methanol as the eluent.

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Product analysis

For rate determinations and routine analyses, products were separated and quantified by GLC-FID on 6 ft column containing 3 % SP2100 on Chromosorb WHP 80–100 mesh with N_2 as carrier gas. A temperature programme of 100 °C for 3 min, 16 °C/min, 180 °C for 3 min was used. Enantiomeric excess was determined by separation of enantiomers on a 50 m x 0.25 mm FS-Lipodex C capillary column (Macherey-Nagel, Duren, Switzerland). The same column was used for GC-MS using a Jeol Ax model 505 mass spectrometer.

Optical rotations (α_D^{25})were determined with a polarimeter (Perkin Elmer model 241) using the products from the scaled-up biotransformation.

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