Comparison of microbiologically and enzymatically mediated Baeyer–Villiger oxidations: synthesis of optically active caprolactones

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Optically active α-substituted caprolactones (7-substituted oxepan-2-ones) were obtained by biocatalysed Baeyer–Villiger oxidations of various α-substituted cyclohexanones using either whole-cells of *Acinetobacter* TD63 or a purified cyclohexanone monooxygenase from *Ps. putida* NCIMB 10007 (MO2). A refinement of the previously established active site model is proposed.

At the present time, the biotransformation approach appears to be, without doubt, the best way to achieve asymmetric Baeyer–Villiger (BV) oxidation,[1] although interesting results have been obtained recently using metal-catalysed reactions.[2] Several examples of such enzymatic oxidation have been described in this context, using either whole-cell cultures[3,4]—which make this biocatalytic approach accessible to the organic chemist—or isolated monooxygenase enzymes purified from bacteria, *i.e. Acinetobacter calcoaceticus*[5] or *Pseudomonas putida* strains.[6,7] The latter approach is particularly useful when several enzymes catalysing the same reaction are present in a single microbial strain (*i.e. Ps. putida*) but are endowed with different (or even opposite) selectivities, thus leading to low optical purity of the product when the whole-cell system is used.

In the course of our previous work, we had investigated the biotransformation of α-substituted cyclopentanones[4,8] using the bacterial strain *Acinetobacter* TD63.[9] This strain was shown to be particularly interesting because, unlike *A. calcoaceticus* NCIMB 9871, it is known to be lacking a lactone hydrolase, an enzyme which begins to metabolize the lactone as soon as it is formed. We observed that the enantioselectivity of these reactions was dependent upon the length of the side chain, and that the lactone of *S*-absolute configuration was preferentially formed in all cases (44% < ee < 97%). Further work on bicyclic compounds,[10] disubstituted cyclohexanones[11] as well as on prochiral cyclobutanones[12] led us to elaborate a model of the enzymatic active site. In contemporaneous work, it has been observed that the bacteria *Ps. putida* is equipped with two different cyclohexanone monooxygenases called respectively MO1 and MO2.[13] The MO1 enzyme was shown to comprise two NADH-dependent enantiocomplementary isozymes whereas MO2 appeared to be NADPH-dependent. The latter enzyme proved to be able to carry out BV oxidations preferentially on monocyclic ketones.[6,13] In some cases,[12] this led to products of opposite absolute configuration to those obtained with the *Acinetobacter* strains. Herein we describe the results obtained by biotransformation of the α-monosubstituted cyclohexanones **1a** to **1g** using whole-cells of *Acinetobacter* TD63 and MO2 enzyme. The aim of this work was to explore the possibility of extending the scope of these biotransformations, to pursue the comparative study of different enzymes, as well as to add more information into our enzyme active site model.

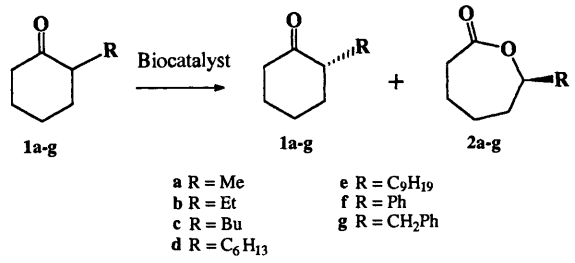

Fig. 1 Biotransformations of α-substituted cyclohexanones by *Acinetobacter* TD63 and MO2

Results and discussion

Biotransformations using whole-cells of *Acinetobacter* TD63

When submitted to a culture of *Acinetobacter* TD63, all α-substituted ketones **1a–g** led to the corresponding ε-caprolactones **2a–g**† (*cf.* Fig. 1). The enantiomeric excesses (ees) of these lactones were determined using chiral GC. (Lipodex E capillary column) or chiral HPLC (Chiralpak AD or Chiralcel OD) analyses and were generally good to excellent. The ees of the remaining ketones were determined either directly using these techniques, or after chemical BV oxidation[14] to the corresponding lactone. The enantiomeric ratio (*E*) values were calculated for each one of the substrates using both the ees of the remaining ketone and that of the lactone according to Sih's equations[15] (*cf.* Table 1) It appears that, whereas this value is low to moderate for **1a**, **1b** and **1e**, values above 100 were obtained for the other substrates, indicating a very high substrate enantioselectivity.

The absolute configurations of the different compounds were determined on the basis of previously published results combined with chemical transformations and chiral GC analysis. Thus, the optical rotation and absolute configuration of the residual ketones **1a**, **1b**, **1f** and **1g** have been previously described.[16,17] The corresponding lactones **2b**, **2f** and **2g** of identical absolute configuration were synthesized from the (optically active) residual ketones by chemical BV oxidation which is known not to alter the absolute configuration of the

† However, the *tert*-butylcyclohexan-2-one was unreactive and stayed unchanged in the medium.

Table 1 Biotransformation of **1a** to **1g** by *Acinetobacter* TD63

	Residual ketone **1**				Lactone **2**				
R	Yield (%)	ee (%)[b] (op %)	$[\alpha]_D^{20}$	Abs. conf'n	Yield (%)	ee (%)[b] (op %)	$[\alpha]_D^{20}$	Abs. conf'n	*E*
a Me	52[a]	35 (32)	−4.5[e] (*c* = 2.6 MeOH)	*R*	35[a]	61 (70)	−17.5[i] (*c* = 1.9 $CHCl_3$)	*S*	6
b Et	10	>98 (92)	−23.1[f] (*c* = 1.7 MeOH)	*R*	60	38	−15.3 (*c* = 2 $CHCl_3$)	*S*	58
c Bu	37[a]	10[c]	Nd	Nd	6[a]	>98	Nd	Nd	>100
d C_6H_{13}	25	35[c]	−6.1 (*c* = 1.5 MeOH)	*R*	23	98	−35.1 (*c* = 1.1 $CHCl_3$)	*S*	>100
e C_9H_{19}	32	42[c]	−7.5 (*c* = 1.9 MeOH)	*R*	26	85	−26.2 (*c* = 1.7 $CHCl_3$)	*S*	20
f Ph	48	86[d] (81)	−92.9[g] (*c* = 0.4 C_6H_6)	*S*	40	>98	+39.5 (*c* = 1.9 $CHCl_3$)	*R*	>100
g CH_2Ph	28	78[d] (73)	−34.4[h] (*c* = 1.2 MeOH)	*S*	22	>96[d]	−45.8 (*c* = 1.3 $CHCl_3$)	*R*	>100

[a] Determined by GC using internal standards [b] Determined by GC (Lipodex E) unless otherwise stated [c] Measured by chiral GC analysis after transformation to lactone by chemical BV reaction. [d] Determined by HPLC (Chiralpak AD or Chiralcel OD). [e] See ref. 12: $[\alpha]_D^*$ +12.2 (*c* = 4, MeOH); (*S*); 87% ee. [f] See ref. 12: $[\alpha]_D^*$ +24.1 (*c* = 4, MeOH); (*S*); 94% ee. [g] See ref. 12: $[\alpha]_D^*$ +114.7 (*c* = 0.45 C_6H_6); (*R*). [h] See ref. 12: $[\alpha]_D^*$ +41.4 (*c* = 5, MeOH); (*R*); 88% ee. [i] See ref. 13: $[\alpha]_D^*$ +25.0 (*c* = 1.8, $CHCl_3$); (*R*). * The temperature used for these measurements has not been provided.

substrate. Comparative chiral GC analysis (Lipodex E) of these synthesised lactones with those of the lactones formed in the course of the bioconversion thus allowed us to assign the *S*-configuration to lactone **2b** and the *R*-configuration to **2f** and **2g**. The *S*-absolute configuration of lactone **2a** was deduced from previously described data.[17] Interestingly, the GC patterns of all the alkyl substituted lactones **2a–d** were similar, *i.e.* the *S*-antipode was always eluted first. This observation has already been emphasised by Fellous *et al.*[18] who showed that the enantiomer interactions with the Lipodex E phase remained the same throughout the series of alkyl ε-caprolactones (alkyl groups: C_nH_{2n+1} with n = 1–8), *i.e.* that no entropic effect that was able to modify the elution order was operating. Thus, this led us to assign tentatively the *S*-absolute configuration to lactones **2c**,‡ **2d**‡ and **2e**, and to deduce those of the residual ketones **1c**, **1d** and **1e** which must be of opposite *R*-configuration.

Examination of our results deserves several comments. It can be seen that all the product lactones—and therefore all the residual ketones—show an identical 'spatial' configuration: *i.e.* the α substituent is always located toward the same side of the space (although there may be some switches in the labelling of the absolute configuration due to the nature of Prelog's priority rule). This very strongly suggests that an identical positioning in the active site is implied for the transformation of these different ketones.

However, some marked differences appear depending on the substrate structure. Thus, kinetic studies showed two types of biotransformation patterns. Methyl, ethyl and phenyl 2-substituted substrates **1a**, **1b** and **1f** reacted rapidly (within 2–3 h) whereas the biooxidation of the other substrates was slower (3–6 h). The time courses of the biooxidation of ketones **1a** and **1b**, as well as those of **1e** and **1g**, are illustrated in Fig. 2. It appears that ketones **1a** and **1b** behaved classically, *i.e.* that the ees of the residual substrates increased whereas those of the corresponding lactones **2a** and **2b** decreased as the reaction proceeded. At the beginning of the reaction, lactones **2a** and **2b** were formed with ees of 73 and 98%, respectively. It is interesting to notice that, in the case of these substrates, the experimental values of the ees, *versus* the conversion ratio, were in perfect agreement with the theoretical curves calculated using Sih's model.[15] This indicates that no secondary reaction and/or no over-metabolism occurred in the course of this reaction. Moreover, lactone formation stopped when cyclohexanediol (the carbon source used for the growth and added as a co-substrate at the beginning of the biotransformation) was completely consumed. Simultaneously, oxygen consumption suddenly slowed down. These observations may be explained by the fact that the cyclohexanediol allows recycling of the NADPH co-factor involved in cellular enzymatic activities.[19] The case of 2-phenylcyclohexanone **1f** is even more interesting, since oxidation stops at about 50% conversion leading to the optically pure lactone (*R*)-**2f** (ee > 98%) and residual ketone **1f** (86% ee). This obviously reflects a high enantioselectivity for this particular substrate.

In the case of the biotransformations of **1c**, **1d**, **1e** and **1g**, the yields of the residual ketone decreased continuously and their ees were moderate, whereas the lactones were formed in low yields but showed excellent ees which stayed constant over the bioconversion period. Simultaneously, small amounts (*ca.* 10%) of the corresponding alcohols were detected. A divergent metabolism—which accounts for the disappearance of the substrate without increase of lactone formation—must be involved. It is interesting that, in all cases, no noticeable 'abnormal' oxygen insertion (which would lead to insertion of the oxygen atom into the less substituted carbon–carbon bond) does occur. Such 'abnormal' lactone formation was described for some other substrates.[10]

Biotransformations using purified MO2 enzyme from *Ps. putida* NCIMB 10007

We have shown previously[6] that the partially purified MO2 monooxygenase from *Ps. putida* grown on (±)-camphor oxidized some α-alkylcyclopentanones into (*S*)-lactones with high ees (>90%) and is more enantioselective than MO1 for monocyclic ketones. In this report, we describe the further purification of MO2 and the biotransformation of substituted α-cyclohexanones **1a** to **1g** using this enzymatic preparation. Thus, MO2 was obtained by ammonium sulfate precipitation and careful ion-exchange chromatography using a Q-Sepharose column, and the extract containing MO2 was checked to be free of MO1 enzyme activity (no oxidation of NADH). The results obtained indicated that, whatever the substituent, all ketones **1a** to **1g** were converted into the corresponding optically active lactones. The yields and ees were generally moderate to good and led to *E* values in the range 2–60 (*cf.* Table 2). The absolute configurations of the formed lactones were (*S*) for alkyl substituents, **2a–e**, and (*R*) for aryl substituents **2f–g**. No 'abnormal' lactones were observed.

Comparison of the results obtained using these two different biocatalysts led to the following observations. Contrary to the case of substituted cyclobutanones,[12] we observed no enantiocomplementarity between these biocatalysts. The absolute configurations of the compounds obtained were the same irrespective of the biocatalyst employed. However, the enantioselectivity (*E*) was generally much higher when the oxidation

‡ Absolute configuration already assigned by Fellous *et al.*[18]

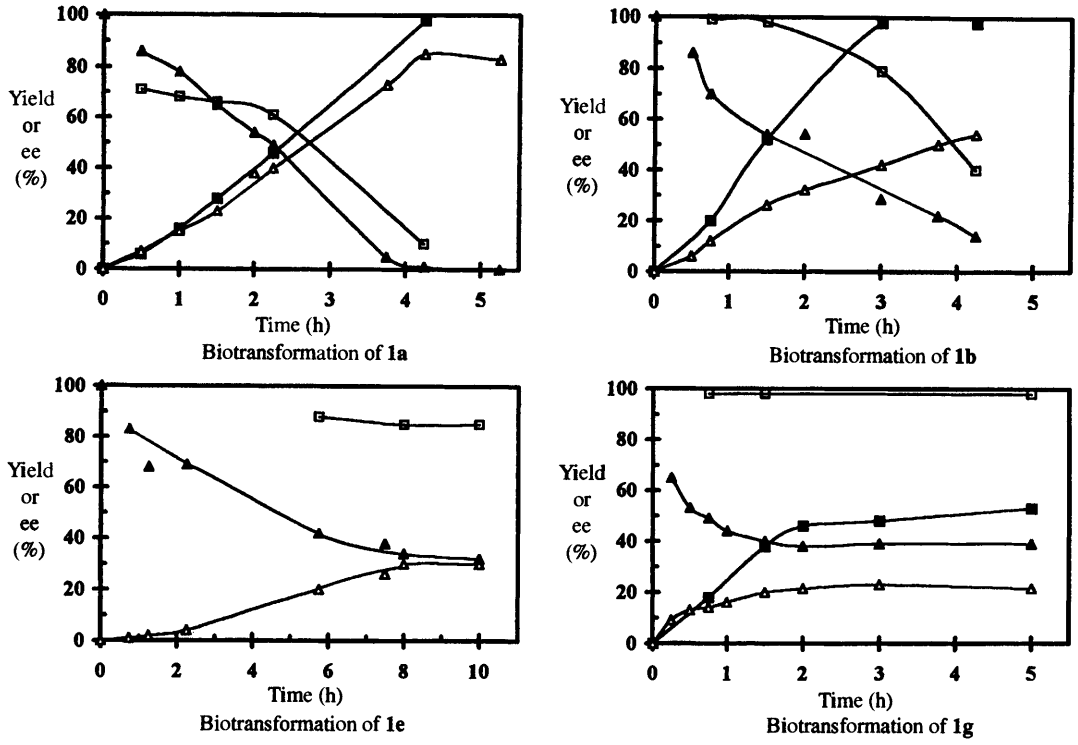

Fig. 2 Time-course of the biotransformations of **1a**, **1b**, **1e** and **1f** by *Acinetobacter* TD63: ▲ = ketone yield; ■ = ketone ee; △ = lactone yield; □ = lactone ee

Table 2 Biotransformation of **1a** to **1g** to purified enzyme MO2

		Residual ketone **1**			Lactone **2**			
R	Reaction time (h)	Yield (%)[a]	ee (%)[b]	Abs. conf'n	Yield (%)[a]	ee (%)[b]	Abs. conf'n	*E*
a Me	1	44	51	*R*	50	38	*S*	4
b Et	1	26	22	*R*	52	25	*S*	2
c Bu	0.5	36	>98	*R*	58	74	*S*	30
d C_6H_{13}	1	54	37	*R*	28	81	*S*	15
e C_9H_{19}	3	75	15[c]	*R*	18	64	*S*	5
f Ph	3	80	17[d]	*S*	13	>98	*R*	60
g CH_2Ph	3	29	83[d]	*S*	66	28[d]	*R*	4

[a] Determined by GC (BP 10) using internal standards. [b] Determined by GC (Lipodex E) unless otherwise stated. [c] Measured by chiral GC analysis after transformation to lactone by chemical BV reaction. [d] Determined by HPLC (Chiralpak AD or Chiralcel OD).

was conducted using the whole cell culture of *Acinetobacter* strain. The ees of the recovered ketones and obtained lactones were higher too. Thus, in this series the best results were obtained using the microbiologically catalysed BV oxidation. The only exception was for ketone **1c**, where the reaction yield was very low with *Acinetobacter* TD63 (6%) but much better with MO2 (58%). As far as cyclopentanones and cyclohexanones are concerned, it appears that MO2 and *Acinetobacter*'s monooxygenase activities are very close in terms of enantioselectivity and regioselectivity.

One of the drawbacks delaying the use of biotransformations in the fine organic chemical industry is obviously the fact that it is very difficult to predict the stereochemical outcome of such transformations, *e.g.* it is difficult to predict the absolute configuration of the reaction products. We have proposed previously a simple model intended to explain, and eventually predict, the regio- and enantio-selectivity of the BV oxidations mediated by *Acinetobacter* sp. strains.[10–12] According to our previous results, we defined a 'forbidden' zone at the upper left corner of our model. Some more information can be gained on the basis of the above results. Thus, by placing the various cyclohexanones **1a** to **1g** into this model following our previously determined rules (*i.e.* considering an equatorial attack of the carbonyl group by the flavine hydroperoxide and a necessary antiperiplanar arrangement of the peroxidic and migrating C–C bond, *cf.* Fig. 3), it appears that four positionings may be adopted by the intermediates obtained from the racemic substrates (*cf.* Fig. 4). This results from the fact that, for each enantiomer of the starting ketone, the substituent may adopt an equatorial or an axial conformation—these conformations being in a rapid equilibrium at least for small substituents.[20] Out of these four possibilities, positionings A and D have to be ruled out, since they would lead to 'abnormal' lactones, the formation of which was not observed. This can be explained by either steric hindrance in the active site (forbidden zone) and/or by the low reactivity of the unsubstituted C–C bond, antiperiplanar with the peroxidic bond in these two positions. This latter hypothesis seems to be consistent with the fact, reported by Taschner,[5] that *cis*-2,6-dimethylcyclohexanone was oxided by cyclohexanone monooxygenase.§ To explain the different enantioselectivities

§ However, it is to be noted that the *Acinetobacter* TD63 strain used in this work is different from the one used by Taschner[5] to isolate cyclohexanone monooxygenase (*A. calcoaceticus* NCIMB 9871), although the results obtained with these two strains have been very similar up to now.

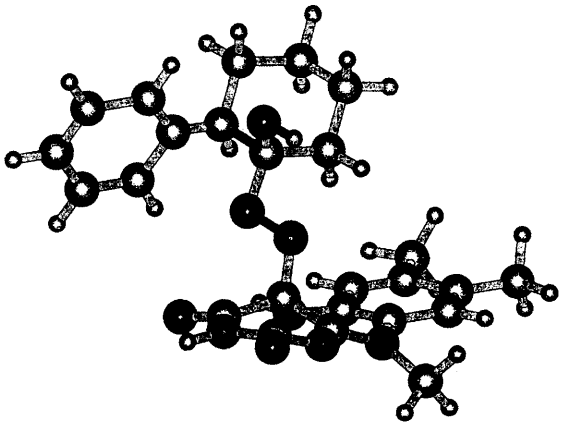

Fig. 3 Hydroxyperoxiflavin intermediate (the ADP group is replaced by a methyl group for clarity)

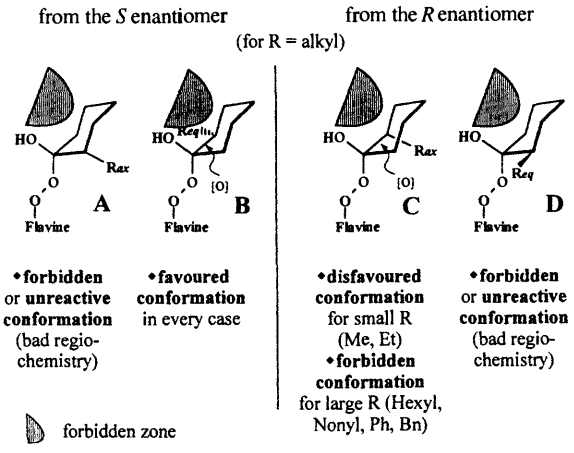

Fig. 4 Different possible positionings for the hydroxyperoxiflavin intermediate

observed, we suppose that position B could be adopted in all cases by one enantiomer (*S* for R = alkyl, *R* for R = Ph or CH_2Ph) and would be favoured, which led us to consider the existence of a large allowed zone, L (*cf.* Fig. 5). Position C, where the substituent is in an axial conformation, could be adopted by ketones **1a** and **1b** (bearing small methyl and ethyl groups) and could be highly disfavoured for substrates **1c** to **1g** (bearing bulkier substituents) because of either steric interactions in the active site (small allowed zone, S) or absence of interconversion between axial and equatorial conformers. Indeed, we observed that the *E* values increased with the size of the substituent. The case of **1e** ($E = 20$) appears, however, as a discrepancy in the series for which we have no explanation at the present time. It is interesting to notice that our model is consistent with another model proposed recently by Ottolina *et al.*[21] (based on cubic-space descriptors and derived from results obtained with sulfides) and with results obtained by Kelly *et al.*[22] with a prochiral tricyclic ketone.

In conclusion, we have shown that α-substituted cyclohexanones may be transformed enantioselectively into the corresponding optically active caprolactones using either whole-cell cultures of *Acinetobacter* TD63 or the MO2 enzyme purified from *Ps. putida* NCIMB 10007. The former procedure affords very straightforward single-step procedures allowing for the preparative-scale synthesis of these very useful chirons. However, in some instances (depending on the nature of the substituent) it is worth using MO2 enzyme as an alternative biocatalyst since this system can lead to better yields of lactone.

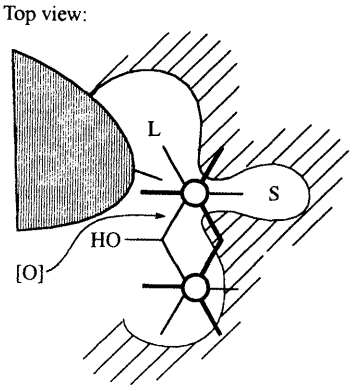

Fig. 5 Top view of the active site model

Experimental

Materials

FID gas chromatography analyses were performed with a Shimadzu GC-14A chromatograph equipped with capillary columns: BP10 (25 m × 0.32 mm × 0.25 μm) and Lipodex® E (25 m × 0.25 mm, Macherey-Nagel). HPLC analyses were performed with a Shimadzu chromatograph equipped with a UV-detector (λ = 220 nm) and Chiralpak-AD or a Chiralcel OD column. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 250 spectrometer in $CDCl_3$ solutions. Chemical shifts (δ) are quoted in ppm with Me_4Si as reference and *J* values are in Hz. Optical rotations were measured on a Perkin-Elmer 241C polarimeter and are given in 10^{-1} deg cm^2 g^{-1}. Biotransformations with *Acinetobacter* TD63 were carried out in a 2 l fermenter (Setric). A phosphate buffer (21 mM pH 7.1) was used for enzymatic purification.

Substrates

Cyclohexanones **1a** and **1f** were obtained from Aldrich. Cyclohexanone **1b** was prepared by oxidation (PDC in CH_2Cl_2, 95% yield) of 2-ethylcyclohexanol. Cyclohexanones **1c**, **1d**, **1e** and **1g** were synthesised in 75–85% yield from cyclohexanone by C-alkylation of the magnesium salts of the cyclohexylimine.[23]

Microorganisms: origin, maintenance and growth

Acinetobacter TD63 was a gift of Professor P. W. Trudgill and *Pseudomonas putida* was obtained from the NCIMB collection (strain number 10007). Stock cultures were grown on nutrient agar slope at 30 °C and then stored at +4 °C. Before biotransformation, *Acinetobacter* TD63[10] and *Ps. putida* NCIMB 10007[6] were grown in the liquid mineral media previously described with, respectively, *cis*/*trans*-cyclohexane-1,2-diol and (±)-camphor as the carbon source.

Purification of MO2 enzyme from *Ps. putida* NCIMB 10007

Growth of the microorganism was carried out as already described.[6] Cells from the culture (2 × 12 l) were harvested at 4000 rpm for 20 min and the cell paste from 24 l of growth medium was resuspended in 600 ml of phosphate buffer (containing PMSF 1 μM and β-mercaptoethanol 3 mM). The cells were disrupted by sonication (4 × 30 s) with 1 min intervals in an ice bath. The dialysed 50–75% $(NH_4)_2SO_4$ fraction, containing both the NADH (MO1) and NADPH (MO2) dependent monooxygenases, was loaded onto a Fast-Flow Q-Sepharose column (2.5 × 20 cm) and eluted with a linear gradient of 0–0.4 M KCl in phosphate buffer. Eluted fractions (6 ml) were assayed measuring substrate-stimulated NADPH oxidation spectrophotometrically at 340 nm. The substrate used was 2-hexylcyclopentanone. Fractions capable of oxidising it were pooled and dialysed to yield a MO2 specific

Table 3 GC and HPLC data for **1** and **2**

	Ketones **1**		Lactones **2**	
	Chiral GC-Lipodex E (1 bar He)			
Compd.	Temp/°C	t_R/min	Temp/°C	t_R/min
a	100 (5 min)–130[a]	3.00 (*S*) 3.14 (*R*)	100 (5 min)–130[a]	15.4 (*S*) 16.3 (*R*)
b	90	4.7 (*S*) 4.9 (*R*)	140	6.2 (*S*) 7.0 (*R*)
c	70	31.6 (*S*) 32.3 (*R*)	130	14.4 (*S*) 15.4 (*R*)
d	80 (60 min)–110[a]	68 (*S*) 70 (*R*)	145	19.8 (*S*) 20.3 (*R*)
e	Nd	Nd	150	67.8 (*S*) 69.9 (*R*)
f	Nd	Nd	160	24.6 (*R*) 25.3 (*S*)
g	115 (58 min)–130[b]	59.9 (*S*) 61.1 (*R*)	130	138 (*S*) 140 (*R*)
	Chiral HPLC (λ = 220 nm, flow rate 1 ml min^{-1})			
	Column Eluent	t_R/min	Column Eluent	t_R/min
f	Chiralcel OD EtOH–Hexane (1/99)	12.0 (*S*) 14.0 (*R*)	Nd	Nd
g	Chiralpak AD EtOH–Hexane (1/99)	9.8 (*R*) 11.7 (*S*)	Chiralpak AD Pr^{i}OH–Hexane (10/90)	6.8 (*S*) 9.7 (*R*)

[a] Temperature rate: 5 °C min^{-1}. [b] Temperature rate: 1 °C min^{-1}.

preparation used for further biotransformations (103 ml, 99 mg protein, 11 U). No oxidation in presence of NADH took place with this preparation.

General procedure for the preparative-scale biotransformations using whole-cells of *Acinetobacter* TD63

A 10 ml starter culture was inoculated from a slope and grown at 30 °C for 7 h. Subsequently, this inoculum was transferred to 1 l of liquid medium[10] in a 2 l fermenter (400 rpm; 30 °C, 0.3 vvm air) and grown for 15 h. Additional cyclohexanediol (0.5 g) and then ketone (1 g) in ethanol (5 ml) were added. The biotransformation was monitored by periodic sampling of aliquots (1 ml) which were extracted by 1 ml of ethyl acetate solution (containing 0.5 g l^{-1} of an internal standard as decane, tetradecane, hexadecane, octadecane or dibutyl phthalate) and analysed by GC (BP10 column). After completion, the biotransformation medium was acidified (pH 1) and extracted with CH_2Cl_2 (continuous extraction, 24 h). The obtained products were purified by flash chromatography (silica gel Merck 60 H; pentane–ether) and bulb-to-bulb distillation.

General procedure for the analytical-scale biotransformations using purified MO2

The ketone (0.5 mg ml^{-1}) in ethanol (0.5%) was stirred (giratory shaker, 200 rpm, 30 °C) with 250–500 µl of the enzymatic preparation in buffer obtained after purification. An equimolar amount of NADPH was added and the reaction was monitored by periodic sampling (0.25 ml) analysed as described for preparative scale.

Identification of products from biotransformations

Using whole-cells of *Acinetobacter* TD63. After purification as described, lactones and ketones were identified by comparison of their ^{1}H and ^{13}C NMR spectra and of their retention times on GC (BP10 and Lipodex E) or on HPLC with literature data and authentic racemic samples prepared by chemical Baeyer–Villiger oxidation (3 equiv. of $NaHCO_3$, 1.1 equiv. of MCPBA in CH_2Cl_2).

For example, 7-nonyloxepan-2-one **2e**: δ_H 4.2 (1 H, m, CH), 2.5–2.2 (2 H, m), 2.1–1.8 (3 H, m), 1.8–1.1 (19 H, m + s) and 0.88 (3 H, t); δ_C 175.8 (CO), 80.74 (CH), 36.55 (CH_2), 35.09 (CH_2), 34.70 (CH_2), 32.00 (CH_2), 29.64 (CH_2), 29.52 (CH_2), 29.41 (2 × CH_2), 28.47 (CH_2), 25.55 (CH_2), 23.20 (CH_2), 22.80 (CH_2) and 14.23 (CH_3).

Using purifed MO2. Lactones and ketones were identified by comparison of their retention times on GC (BP10 and Lipodex E) or on HPLC with authentic samples.

Measurements of the enantiomeric excesses of lactones and optically active residual ketones. The ees of lactones **2a–g** and ketones **1a–d** were determined directly by chiral GC (Lipodex E column) and those of compounds **1f**, **1g** and **2g** by chiral HPLC (Chiralcel OD or Chiralpak AD) using racemic compounds as reference standard. Moreover, all ketones were transformed by chemical BV oxidation to the corresponding lactones which were analysed by chiral chromatography. The analysis conditions are described in Table 3.

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