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Biooxidation of ketones with a cyclobutanone structural motif by recombinant whole-cells expressing 4-hydroxyacetophenone monooxygenase

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Abstract

Whole cells of an *Escherichia coli* strain overexpressing 4-hydroxyacetophenone monooxygenase (HAPMO) originating from *Pseudomonas fluorescens* ACB have been used to study the stereopreference in the Baeyer–Villiger reaction of ketones bearing a cyclobutanone structural motif. The enzyme catalyzes the oxidation of several prochiral cyclobutanones to antipodal butyrolactones as obtained in cyclohexanone monooxygenase mediated conversions. However, chemical yields and enantiomeric purity are usually moderate. The regioselectivity in oxidations of fused cylobutanones differs from the previously observed behavior for Baeyer–Villiger monooxygenases with highly varying ratios of "normal" and "abnormal" product lactones and enantioselectivities. Taken together with previous substrate profiling data, HAPMO represents a distinctively different biocatalytic entity compared to monooxygenases characterized so far.

Keywords: 4-Hydroxyacetophenone monooxygenase; Baeyer–Villiger oxidation; Cyclobutanones; Butyrolactones; Whole-cell biotransformation

1. Introduction

Oxidations still represent a key challenge for the development of sustainable processes compatible with the concept of "green chemistry" and simultaneously implementing highest safety standards. An armament of highly diverse enzymes can be offered for various oxygen incorporation reactions and this strategy is highly compatible with the above prerequisites [1]. One of the representative domains to demonstrate the versatility and potential of biotransformations is the Baeyer–Villiger oxidation [2], in particular when it is carried out in enantioselective manner [3]. Baeyer–Villiger monooxygenases (BVMOs) were established in recent years as catalytic entities with a remarkably broad substrate tolerance and specificity for this oxygen insertion process [4].

By exploiting previous advances in molecular biology, an increasing number of novel BVMOs originating from various natural sources have been identified over the past years [5–7]. Together with recent attempts to modify the stereospecificity of such enzymes [8], the characterization of these enzymes is becoming a key aspect in order to evaluate their potential as biocatalysts in organic synthesis.

Recently, 4-hydroxyacetophenone monooxygenase (HAPMO) was described as a novel BVMO with an interestingly different substrate specificity [9] compared to extensively studied cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NICMB 9871 [10]. This

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enzyme displays high catalytic activity for the conversion of aryl and hetaryl ketones and moderate acceptance of aliphatic ketones. Furthermore, the monooxygenase performs highly enantioselective sulfoxidations of aryl sulfides [9b]. With the exception of fused bicycloketone **3a**, no cycloketones were described as substrates, so far.

With this study we are closing this gap and present our results for the biooxidation of precursors bearing a cyclobutanone structural motif utilizing this newly identified BVMO. The corresponding lactones represent interesting intermediates for the synthesis of natural products and bioactive compounds. Butyrolactones **2** derived from prochiral 3-substituted cyclobutanones **1** can be used as precursors for the synthesis of various lignans such as enterolactone, hinokinin and arctigenin [11] (Scheme 1). Such compounds can also serve as platforms for the subsequent synthesis of β-amino acids [12], GABA inhibitors such as baclofen [13] or analgesics like eptazocine [14].

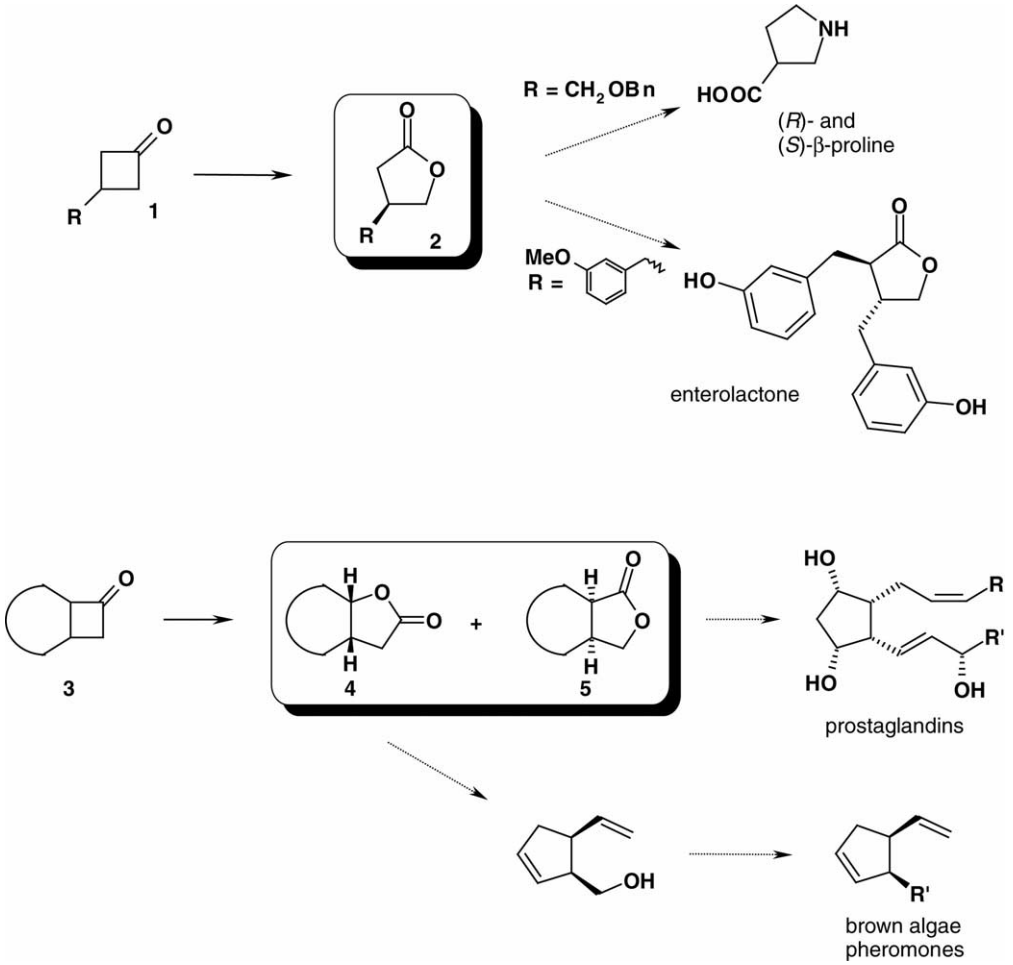
While biotransformations of such prochiral substrates generate the required lactones in a 100% theoretical yield, racemic starting material can undergo a kinetic resolution process. In the case of fused bicycloketones **3** complete conversion to two regioisomeric lactones **4** ("normal") and **5** ("abnormal") was observed with several enzymes [15,16]. Both optically pure products are key intermediates for the synthesis of prostaglandins [17], brown algae pheromones [18] and cytostatics (sarkomycin) [19], as outlined in Scheme 1.

In order to circumvent the obstacles of enzyme isolation and coenzyme recycling, a recombinant whole-cell overexpression system was utilized in this study. In recent years, this strategy was successfully employed in synthetic applications of several BVMOs from various native origin utilizing *S. cerevisiae* [20] and *E. coli* [21–23] (as in this project) as suitable expression hosts.

2. Experimental

2.1. General

Unless otherwise noted, chemicals and microbial growth media were purchased from commercial suppliers and used without further purification. All solvents were distilled prior



Scheme 1. Desymmetrization and regiodivergent Baeyer–Villiger oxidation of prochiral cyclobutanones **1** and fused ketones **3** to key intermediates for the synthesis of various bioactive compounds and natural products.

to use. Shake flask fermentations were performed in a Gerhard THO5 orbital thermoshaker. Flash column chromatography was performed on silica gel 60 from Merck (40–63 μm). Preparative MPLC was carried out on a Büchi 681/684 system with silica gel packed columns. Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. NMR-spectra were recorded from $CDCl_3$ or DMSO-d_6 solutions on a Bruker AC 200 (200 MHz) or Bruker Avance Ultra-Shield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using TMS as internal standard. Enantiomeric excess was determined by chiral phase GC analysis using a BGB 173 or BGB 175 column (30 m × 0.25 mm i.d., 0.25 μm film) on a HP 6890 series chromatograph and compared to racemic reference material obtained by chemical oxidation (*m*-CPBA) where applicable. Biotransformation progress and conversion control were performed with a ThermoQuest Trace GC 2000 using a standard capillary column DB5 (30 m × 0.32 mm i.d.). Specific rotation $[\alpha]_D^{20}$ was determined using a Perkin-Elmer Polarimeter 241.

2.2. Bacterial strains and growth

E. coli TOP10 containing the structural gene for HAPMO on a pBAD vector was routinely cultivated on LB-agar (1% Bacto–Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl, 1.5% agar) plates supplemented by ampicillin (200 μg/mL) and stored as frozen stocks (addition of glycerol to a final concentration of 15%) at −80 °C.

Liquid cultures were grown in standard LB media (1% Bacto–Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl) supplemented by ampicillin in baffled Erlenmeyer flasks on an orbital shaker (120 rpm at 37 °C). Protein expression was induced at 25 °C by addition of arabinose to a final concentration of 0.002% (w/v).

2.3. Cyclobutanone substrates

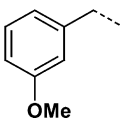
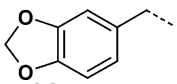
Prochiral cyclobutanone substrates were prepared according to the literature either by Cu/Zn couple mediated cyclization under thermal [24] or ultrasound [25] conditions from the corresponding alkenes and trichloroacetyl chloride. Subsequent dechlorination gave the required ketone substrates.

With the exception of commercially available ketone **3a**, fused bicycloketones were prepared according to a [2+2] cycloaddition and dehalogenation sequence from the corresponding cyclic alkenes [25].

2.4. Biotransformations

Fresh LB-ampicillin medium was inoculated with an aliquot (1%, v/v) of an overnight preculture of recombinant *E. coli* in a baffled Erlenmeyer flask. The culture was shaken at 120 rpm at 37 °C for 2 h until it reached an optical density at 600 nm (OD_{600}) between 0.2 and 0.4. Then HAPMO production was induced by adding arabinose, the substrate (3–5 mM) was added neat together with β-cyclodextrin[26] (1 equiv.) and the culture was continuously shaken at 120 rpm at room temperature. Conversions were monitored by GC and typically required between 12 and 24 h to reach completion. The culture was centrifuged to remove the cells and eventually (in case of incomplete separation of cell material) passed through a bed of Celite®. The aqueous layer was saturated with sodium chloride and extracted with ethyl acetate. In the case of ketones **3e–g**, the aqueous layer was not saturated with sodium chloride and extracted with dichloromethane. Extractions were usually carried out with approximately equal volumes of organic solvent (to avoid emulsions) and repeated until complete recovery of the products (checked by TLC). The combined organic layers were dried over anhydrous sodium

Table 1
Desymmetrization of prochiral cyclobutanones **1**

R	Ketones		Lactones				
		Starting material		Yield[a]	e.e. (absolute configuration)[b]	$[\alpha]_D^{20}$	References
Ph	**1a**	100 μL, 110 mg	**2a**	12% (14.1 mg)	92% (*S*)	+42.6 (*c* 0.28, MeOH)	[27,28]
Bn	**1b**	100 μL, 106 mg	**2b**	26% (29.8 mg)	44% (*R*)	+2.7 (*c* 0.52, $CHCl_3$)	[11,28,29]
	**1c**	100 μL, 108 mg	**2c**	42% (49.3 mg)	56% (*R*)	+2.6 (*c* 0.99, $CHCl_3$)	[11,28]
	**1d**	100 μL, 93.5 mg	**2d**	63% (63.9 mg)	66% (*R*)	+2.4 (*c* 1.28, $CHCl_3$)	[11,28,29]
$BnOCH_2$	**1e**	100 μL, 116 mg	**2e**	35% (44 mg)	29% (*R*)	−8.8 (*c* 1, $CHCl_3$)	[28,29]
Bu	**1f**	200 μL, 150 mg	**2f**	52% (88 mg)	44% (*S*)	−2.8 (*c* 1, $CHCl_3$)	[29]
i-Bu	**1g**	100 μL, 94 mg	**2g**	31% (32.7 mg)	13% (*S*)	−0.46 (*c* 0.65, $CHCl_3$)	[29]

[a] Isolated yield of purified product.
[b] e.e. determined by chiral phase GC.

sulfate, filtered and concentrated in vacuo. The crude lactones were purified by flash column chromatography or MPLC and subsequently analyzed by NMR and chiral phase GC.

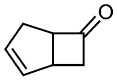
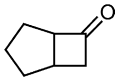
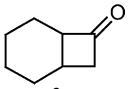
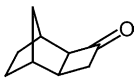
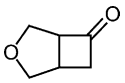
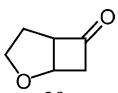
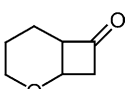
3. Results and discussion

Desymmetrization of prochiral cyclobutanones **1a–g** with recombinant *E. coli* was compared to transformations with a similar expression system for cyclohexanone monooxygenase (CHMO) from *Acinetobacter* [21a]. The assignment of absolute configuration for lactones **2a–g** is based on the literature [27–29]. Conversion with HAPMO producing cells usually gave moderate to acceptable yields of the expected lactones with minor amounts of indole as product contamination (Table 1). Lactones **2** were generally purified by preparative chromatography and analyzed by NMR and chiral phase GC. Precursor **1a** was the only exception, where whole-cell mediated biotransformations gave significant amounts of a by-product. In this case, a 1:2 mixture of lactone **2a** and 3-phenylcyclobutanol was isolated upon fermentation work-up, which was rather difficult to separate. Consequently, a rather low yield for compound **2a** was obtained, however, in good optical purity. We attribute formation of the alcohol to an unexpected reductase activity from the host *E. coli* strain with a remarkable reactivity for substrate **1a**.

Benzyl-substituted precursors **1b–d** as well as ether **1e** were readily converted by HAPMO producing cells. It is striking, that biotransformations with HAPMO produced lactones **2a–e** bearing an aromatic ring in antipodal form to previous biooxidations using whole-cells of native bacteria (*Acinetobacter*) or fungi (*Cunninghamella*) [28]. This enantiocomplementary oxidation was not observed for ketones **1f/g** with aliphatic substitutents.

Stereospecificity of the enzyme seems to depend on the distance of sterically demanding groups relative to the reactive carbonyl center. While a phenyl substituent (**1a**) induces good enantioselectivity, optical purity for benzyl substituted precursors (**1b–d**) with an additional methylene spacer is significantly decreased. Further increase of the spacer between the prochiral center and the phenyl group (**1e**) or a straight as well as branched alkyl chain (**1f/g**) possess comparably inefficient potential to induce chirality.

Table 2
Regiodivergent oxidation of racemic ketones **3**

Ketone	Starting material	Total yield[a]	"Normal" lactones **4a–g**		Ratio	"Abnormal" lactones **5a–g**	
			e.e.(%)[b]	Absolute configuration		e.e. (%)[b]	Absolute configuration
 **3a**	50 μL, 51 mg	56% (33 mg)	75	1*S*, 5*R*	32:68	32	1*R*, 5*S*
 **3b**	50 μL, 51 mg	53% (31 mg)	36	1*S*, 5*S*	45:55	29	1*R*, 5*S*
 **3c**	50 μL, 55 mg	56% (34 mg)	15	1*S*, 6*S*	87:13	99	1*S*, 6*R*
 **3d**	50 μL, 51 mg	79% (45 mg)	0	N.D.	58:42	95	N.D.
 **3e**	50 μL, 58 mg	63% (42 mg)	71	1*R*, 5*R*	31:69	37	1*S*, 5*R*
 **3f**	50 μL, 58 mg	62% (41 mg)	59	1*S*, 5*S*	15:85	6	1*R*, 5*S*
 **3g**	50 μL, 54 mg	79% (48 mg)	52	1*S*, 6*S*	90:10	>99	1*S*, 6*R*

[a] Combined isolated yield of **4** and **5** after purification by flash column chromatography.
[b] e.e. determined by chiral phase gas chromatography.

Biooxidation of compound **3a** is one of the "benchmark" reactions for BVMO performance. In the case of CHMO from *Acinetobacter*, biotransformation to a 1:1 mixture of lactones **4** and **5** is observed in high stereoselectivity.

An array of carbo- and heterocyclic substrates **3a–g** [15,25,30] was studied to investigate the effects of ring size, hybridization, and polarity on the biotransformation. Microbial Baeyer–Villiger oxidation of ketones **3a–g** with recombinant *E. coli* expressing HAPMO gave complete conversion of substrates to lactone products in all cases. A mixture of lactones **4** and **5** was obtained after preparative chromatography and analyzed by chiral phase GC. Biotransformation results are summarized in Table 2. Assignment of absolute configuration is based on biotransformations with recombinant *E. coli* expressing CHMO and literature data [15]. Separation of lactones **4** and **5** can be accomplished by repeated column chromatography and physical properties for these compounds agreed with published data.

Biooxidation of compound **3a** has been studied for HAPMO, recently, using isolated enzyme [9b]. Compatible with this study, we observed predominant formation of "abnormal" lactone **5a** in low stereoselectivity by whole cells transforming racemic substrate. "Normal" lactone **4a** was obtained in higher enantiomeric excess. A similar trend for product distribution was obtained for all five-membered precursors **3a/b** and **3e/f** favoring formation of the corresponding "abnormal" lactone. In all cases, the e.e. of **5** was significantly lower than **4**.

A different situation was observed for six-membered ketones **3c/d** and **3g**. Here, the major product was the "normal" lactone **4**. Consequently, enantiomeric excess for this compound was low to moderate compared to the corresponding "abnormal" lactone **5**. In all cases the stereopreference for the prepared lactones was comparable to data obtained with CHMO from *Acinetobacter*.

4. Conclusion

The above results for the regiodivergent oxidation of bicycloketones are remarkable in the context of our previous attempts to classify BVMOs by their biocatalytic performance. Recently, we have found a significantly different behavior of two enzymes from *Brevibacterium* for this biooxidation [16]. One of these enzymes behaves similar to CHMO from *Acinetobacter* giving clean regiodivergent oxidation in high optical purity. The second BVMO predominantly yields "normal" lactones **4** with low e.e. in several cases. Taken together with previous findings about enantiodivergent oxidation of prochiral ketones by the enzyme CPMO from *Comamonas*, the *Acinetobacter* protein and the two BVMOs from *Brevibacterium* a certain trend could be demonstrated, which is supported by sequence data [22,23].

HAPMO on the other hand displays a distinctly different substrate specificity and stereopreference for both desymmetrizations and regiodivergent oxidations. These unique properties of the biocatalyst are confirmed by its position in a comprehensive phylogenetic tree analysis of a multitude of flavin dependent monooxygenases [5], as it has only a very remote relation to the other four proteins.

Although enantioselectivites of products obtained by biooxidation with HAPMO do not attain values of the commonly utilized BVMOs, so far, some quite unusual biocatalytic properties of the enzyme were discovered in the context of this project. This might serve as a starting point to further investigate the potential of this enzyme, as fine tuning of novel catalytic features is becoming more and more laboratory routine with the modern armament of molecular biology, demonstrated recently for CHMO [8]. Considering the enormous number of monooxygenases yet unstudied with respect to their biocatalytic performance, substantial progress can be expected in this field in years to come.

Acknowledgements

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References

[1] (a) Z. Li, J.B. van Beilen, W.A. Duetz, A. Schmid, A. de Raadt, H. Griengl, B. Witholt, Curr. Opin. Chem. Biol. 6 (2002) 136;
(b) S.G. Burton, Trends Biotechnol. 21 (2003) 543.
[2] (a) G.R. Krow, Org. React. 43 (1993) 251;
(b) M. Renz, B. Meunier, Eur. J. Org. Chem. (1999) 737.
[3] M.D. Mihovilovic, F. Rudroff, B. Grötzl, Curr. Org. Chem. 8 (2004) 1057.
[4] (a) N.M. Kamerbeek, D.B. Janssen, W.J.H. van Berkel, M.W. Fraaije, Adv. Synth. Catal. 345 (2003) 667;
(b) M.D. Mihovilovic, B. Müller, P. Stanetty, Eur. J. Org. Chem. (2002) 3711;
(c) S.M. Roberts, P.W.H. Wan, J. Mol. Catal. B: Enzym. 4 (1998) 111.
[5] (a) M.W. Fraaije, N.M. Kamerbeek, W.J.H. van Berkel, D.B. Janssen, FEBS Lett. 518 (2002) 43;
(b) M.W. Fraaije, N.M. Kamerbeek, A.J. Heidekamp, R. Fortin, D.B. Janssen, J. Biol. Chem. 279 (2004) 3354.
[6] J.B. van Beilen, F. Mourlane, M.A. Seeger, J. Kovac, Z. Li, T.H.M. Smits, U. Fritsche, B. Witholt, Environ. Microbiol. 5 (2003) 174.
[7] (a) P.C. Brzostowicz, K.L. Gibson, S.M. Thomas, M.S. Blasko, P.E. Rouviere, J. Bacteriol. 182 (2000) 4241;
(b) P. Brzostowicz, D.M. Walters, S.M. Thomas, V. Nagarajan, P.E. Rouviere, Appl. Environ. Microbiol. 69 (2003) 334.
[8] M.T. Reetz, B. Brunner, T. Schneider, F. Schulz, C.M. Clouthier, M.M. Kayser, Angew. Chem. 116 (2004) 4167.

[9] (a) N.M. Kamerbeek, M.J.H. Moonen, J.G.M. van der Ven, W.J.H. van Berkel, M.W. Fraaije, D.B. Janssen, Eur. J. Biochem. 268 (2001) 2547;
(b) N.M. Kamerbeek, A.J.J. Olsthoorn, M.W. Fraaije, D.B. Janssen, Appl. Environ. Microbiol. 69 (2003) 419;
(c) M.C. Gutierrez, V. Alphand, R. Furstoss, J. Mol. Catal. B: Enzym. 21 (2003) 231.
[10] J.D. Stewart, Curr. Org. Chem. 2 (1998) 211.
[11] J.W. Bode, M.P. Doyle, M.N. Protopopova, Q.-L. Zhou, J. Org. Chem. 61 (1996) 9146.
[12] C. Mazzini, J. Lebreton, V. Alphand, R. Furstoss, J. Org. Chem. 62 (1997) 5215.
[13] C. Mazzini, J. Lebreton, V. Alphand, R. Furstoss, Tetrahedron Lett. 38 (1997) 1195.
[14] S. Shiotani, H. Okada, T. Yamamoto, K. Nakamata, J. Adachi, H. Nakamoto, Heterocycles 43 (1996) 113.
[15] V. Alphand, R. Furstoss, J. Org. Chem. 57 (1992) 1306;
F. Petit, R. Furstoss, Tetrahedron: Asymmetry 4 (1993) 1341.
[16] M.D. Mihovilovic, P. Kapitan, Tetrahedron Lett. 45 (2004) 2751.
[17] V. Alphand, A. Archelas, R. Furstoss, Tetrahedron Lett. 30 (1989) 3663.
[18] J. Lebreton, V. Alphand, R. Furstoss, Tetrahedron Lett. 37 (1996) 1011;
J. Lebreton, V. Alphand, R. Furstoss, Tetrahedron 53 (1997) 145.
[19] L. Andrau, J. Lebreton, P. Viazzo, V. Alphand, R. Furstoss, Tetrahedron Lett. 38 (1997) 825.
[20] (a) J.D. Stewart, K.W. Reed, M.M. Kayser, J. Chem. Soc., Perkin Trans. 1 (1996) 755;
(b) J.D. Stewart, W.R. Kieth, J. Zhu, G. Chen, M.M. Kayser, J. Org. Chem. 61 (1996) 7652;
(c) M.M. Kayser, G. Chen, J.D. Stewart, J. Org. Chem. 63 (1998) 7103.
[21] (a) G. Chen, M.M. Kayser, M.D. Mihovilovic, M.E. Mrstik, C.A. Martinez, J.D. Stewart, New J. Chem. 23 (1999) 827;
(b) S.D. Doig, L.M. O'Sullivan, S. Patel, J.M. Ward, J.M. Woodley, Enzyme Microb. Technol. 28 (2001) 265.
[22] (a) H. Iwaki, Y. Hasegawa, P.C.K. Lau, S. Wang, M.M. Kayser, Appl. Environ. Microbiol. 68 (2002) 5681;
(b) M.D. Mihovilovic, B. Müller, A. Schulze, P. Stanetty, M.M. Kayser, Eur. J. Org. Chem. (2003) 2243.
[23] M.D. Mihovilovic, F. Rudroff, B. Müller, P. Stanetty, Bioorg. Med. Chem. Lett. 13 (2003) 1479.
[24] B.D. Johnston, E. Czyzewska, A.C. Oehlschlager, J. Org. Chem. 52 (1987) 3693.
[25] G. Mehta, P.S.H. Rao, Synth. Commun. 15 (1985) 991.
[26] R. Bar, Trends Biotechnol. 7 (1989) 2.
[27] M. Sato, Chem. Pharma. Bull. 29 (1981) 2885.
[28] V. Alphand, C. Mazzini, J. Lebreton, R. Furstoss, J. Mol. Catal. B: Enzym. 5 (1998) 219.
[29] R. Gagnon, G. Grogan, E. Groussain, S. Pedragosa-Moreau, P.F. Richardson, S.M. Roberts, A.J. Willetts, V. Alphand, J. Lebreton, R. Furstoss, J. Chem. Soc. Perkin Trans. 1 (1995) 2527.
[30] L. Ghosez, R. Montaigne, A. Roussel, H. Vanlierde, P. Mollet, Tetrahedron 27 (1971) 615.