ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 349–353

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Baeyer-Villiger oxidations of representative heterocyclic ketones by whole cells of engineered *Escherichia coli* expressing cyclohexanone monooxygenase

Marko D. Mihovilovic [a,*], Bernhard Müller [a], Margaret M. Kayser [b], Jon D. Stewart [c,1], Johannes Fröhlich [a], Peter Stanetty [a], Helmut Spreitzer [d]

[a] *Institute for Organic Chemistry, Vienna University of Technology, Getreidemarkt 9, A-1060 Vienna, Austria*
[b] *Department of Physical Sciences, University of New Brunswick, Saint John, N.B., E2L 4L5, Canada*
[c] *Department of Chemistry, University of Florida, Gainesville, FL 32611, USA*
[d] *Institute of Pharm. Chemistry, Vienna University, A-1090 Vienna, Austria*

Abstract

Whole cells of an *Escherichia coli* strain overexpressing *Acinetobacter* sp. NCIB 9871 cyclohexanone monooxygenase (CHMO; E.C. 1.14.13.22) have been used for the Baeyer-Villiger oxidation of representative heterocyclic six-membered ketones to probe the potential impact of nitrogen, sulfur and oxygen on the chemoselectivity of these reactions. The fact that all of these heterocyclic systems were accepted as substrates by the enzyme and gave normal Baeyer-Villiger products broadens the synthetic utility of the engineered *E. coli* strain and emphasizes the chemoselectivity achievable with enzymatic oxidation catalysts.

Keywords: Cyclohexanone monooxygenase; Baeyer-Villiger oxidation; Chemoselective oxidation; Heterocyclic ketones; Whole-cell biotransformation

1. Introduction

A variety of enzymes catalyzing Baeyer-Villiger oxidations have been described in recent years [1]. Usually these enzymes are part of degradative pathways in microorganisms that enable the use of non-carbohydrate compounds as sources of carbon and cell energy [2,3]. Such enzymes very often display a remarkably broad substrate acceptance, even though degradation is usually associated with a specific compound. Furthermore, Baeyer-Villigerase enzymes often exhibit high enantioselectivities. These two attributes make these oxidases useful catalysts for asymmetric synthesis, complementing recent advances in this area based on chiral metal complexes [4–8].

Cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIB 9871 (E.C. 1.14.13.22) [9] is the best-known Baeyer-Villiger enzyme to date and it has been shown to oxidize more than 80 different ketones [1]. The enzyme has been expressed in both baker's yeast (*Saccharomyces cerevisiae*)

* Corresponding author.
E-mail address: mmihovil@pop.tuwien.ac.at (M.D. Mihovilovic).

[1] Camille and Henry Dreyfus Foundation New Faculty Awardee, 1994–1999.

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00021-7

and *Escherichia coli* to provide whole cells that can be used directly as synthetic reagents, removing the need to isolate the enzyme and provide a generation system for reduced nicotinamide adenine dinucleotide phosphate (NADPH) [10–14]. These two engineered strains make CHMO accessible to nonspecialists and provide simple means to synthesize a variety of chiral building blocks.

The engineered *E. coli* strain was originally created to oxidize a series of thioethers to chiral sulfoxides without side-reactions caused by host-encoded enzymes [14]. Its high efficiency in these reactions suggested that it might also be useful for Baeyer-Villiger oxidations of ketones. We were particularly interested in heterocyclic ketones, since those with nitrogen or sulfur pose chemoselectivity challenges that had not been explored systematically prior to our work, although Latham and Walsh [15] showed that 3- and 4-thiacyclohexanones were oxidized by purified CHMOs to the corresponding ε-caprolactones without competing oxidation at sulfur.

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 to use. Flash column chromatography was performed on silica gel 60 from Merck (40–63 μm). *Kugelrohr* distillation was carried out using a Büchi GKR-51 apparatus. Melting points were determined using a Kofler-type Leica Galen III micro hot stage microscope and are uncorrected. Elemental analyses were carried out in the Microanalytical Laboratory, University of Vienna. GC/MS was performed on a HP MSD 5890/5970 unit using a HP-5MS cross linked 5% PH ME siloxane column (30 m × 0.25μm, HP Part No. 19091S-433). The NMR spectra were recorded from $CDCl_3$ solutions on a Bruker AC 200 (200 MHz) or Bruker Avance UltraShield 400 (400 MHz) spectrometer and chemical shifts are reported in ppm using Me_4Si as internal standard. Peak assignment is based on correlation experiments.

2.2. Substrates and reference material

The heterocyclic substrates were either obtained from commercial suppliers (**1b**, **1c**) or synthesized according to literature procedures (**1a**) [16]. Compound **1d** was prepared via ketalization of commercially available 4-piperidone hydrate hydrochloride (**1g**, X = NH) with ethylene glycol [17] alkylation with allylbromide in the presence of potassium carbonate [18] followed by deketalization using concentrated HCl [19]. Acylation of ketones **1e** and **1f** were performed via standard protocols by conversion of **1g** with the corresponding acid chlorides in dichloromethane solution in the presence of triethylamine or pyridine as base.

Where possible (**1b**, **1e**, **1f**) reference samples of the expected lactones were prepared by treatment of the corresponding ketones with *m*-chloroperbenzoic acid (*m*-CPBA) in dichloromethane solution at room temperature overnight [20]. Pure products were obtained after flash column chromatography or *Kugelrohr* distillation.

2.3. Bacterial strains and growth

Plasmid pMM4 contains the structural gene for CHMO from *Acinetobacter* sp. NCIB 9871 on a pET22b(+)-based plasmid along with an ampicillin resistance marker. Its construction has been described in detail recently [14]. Expression of CHMO utilizes the highly efficient T7 promoter [21] and is induced in the engineered *E. coli* strain [BL21-(DE3)(pMM4)] by adding isopropylthio-β-D-galactoside (IPTG) to the growth medium, which allows accumulation of CHMO up to 20% of total protein after a few hours.

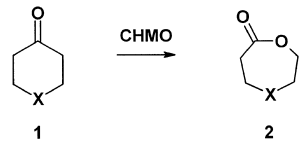
Cells of BL21(DE3)(pMM4) were routinely cultured in LB medium (1% Bacto-Peptone, 0.5% Bacto-Yeast Extract, 1% NaCl, all obtained from Amersham Life Science) containing ampicillin (200 μg/ml) at 37°C with shaking at 120 rpm. Frozen stocks (1 ml portions) were prepared by adding glycerol (final concentration 15%) to a culture grown overnight and stored at −80°C. Fresh plates were streaked weekly from frozen stocks and grown overnight at 37°C on LB-ampicillin plates containing 1.5% Bacto-Agar.

2.4. Biotransformations and product isolation

Fresh LB-ampicillin medium (250 ml) was inoculated with a 2.5 ml aliquot of an overnight preculture of BL21(DE3)(pMM4) in a 1000 ml Erlenmeyer flask. The culture was shaken at 120 rpm at 37°C until it reached an optical density at 600 nm (OD_{600}) between 0.2 and 0.4, then IPTG was added to a final concentration of 0.025 mM. The substrate (100 mg) was added neat and β-cyclodextrin (1 eq.) was supplemented if required. The culture was shaken at 150 rpm at room temperature. Complete conversions required between 10 and 18 h, after which time the culture was passed through a bed of Celite® to collect the cells, which were washed subsequently with dichloromethane. The aqueous filtrate was saturated with sodium chloride and the organic layer was separated. The aqueous layer was extracted twice with equivalent volumes of dichloromethane. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated. The crude products were purified by flash column chromatography or *Kugelrohr* distillation.

3. Results and discussion

Initial test experiments were performed with ketone **1a** since this compound was already described as a substrate for isolated CHMO [15]. The best results for fermentations in shake flasks were obtained with an initial ketone concentration of approx. 3 mM (Table 1, entry 1). No oxidation of the thioether functionality was observed even after prolonged exposure to growing cultures of the recombinant strain. Control experiments with the unmodified strain BL21(DE3) gave no formation of lactone **2a**, indicating that CHMO was responsible for the Baeyer-Villigerase activity of the engineered *E. coli* strain (Scheme 1).



Scheme 1.

The ability of CHMO to convert heterocyclic ketones to the corresponding ε-caprolactones was investigated further by testing ketones **1b**–**f** as potential substrates for biotransformations by the engineered *E. coli* strain. Using standard conditions, ketone **1b** gave an excellent yield of **2b**, whose properties were identical to those prepared by *m*-CPBA oxidation (entry 2). The same enzyme also accepted 4-piperidones. For example, *N*-methylpiperidone **1c** was oxidized to the corresponding **2c** (entry 3). This result is noteworthy since reagents such as peracids yield *N*-oxides rather than Baeyer-Villiger products [22,23]. Methods to cleave the *N*-methyl substituent from lactones such as **2c** have been reported.[2]

Oxidations of other *N*-substituted 4-piperidones were explored in order to provide lactones that could be deprotected under milder conditions. While an *N*-allyl substituent as potential protecting group[3] was tolerated (**1d**), complete conversion could not be achieved under the biotransformation conditions (entry 4). Lactone **2d** was obtained in only small quantities and characterization relied on its mass spectrum. By contrast, both *N*-acetyl substituted 4-piperidinones (ketone **1e**, entry 5) and *N*-methyl carbamoyl-substituted 4-piperidinones (ketone **1f**, entry 6) were good substrates and the expected lactones were isolated in good yields. These materials were identical to authentic samples obtained via classical Baeyer-

Table 1

Entry	Ketone	X	Lactone	Yield[a]
1	**1a**	S	**2a**	48%
2	**1b**	O	**2b**	79%
3	**1c**	NMe	**2c**	50%
4	**1d**	$NCH_2CH{=}CH_2$	**2d**	10%[b]
5	**1e**	NCOMe	**2e**	39%[c] (59%)[d]
6	**1f**	NCOOMe	**2f**	40%[c] (67%)[d]

[a] Isolated yield of purified product.
[b] Yield determined by GC, predominantly starting material recovered.
[c] β-Cylodextrin added to improve conversion.
[d] Yield based on recovered starting material.

[2] For a variety of methods for *N*-demethylations, refer to Refs. [24–26] and references therein.
[3] For a review about the allyl protecting group in general, see Refs. [27,28].

Villiger oxidation with peracids. Both of these protecting groups can be cleaved under a variety of conditions.[4] Unfortunately, the high polarities of ketones **1e** and **1f** caused some difficulties in achieving complete conversions. Although the addition of β-cyclodextrin improved conversion (a known effect of cyclic sugars of this type [30]), some starting material was still recovered at the conclusion of the biotransformation. In conclusion, these experiments show that CHMO tolerates the presence of electron withdrawing nitrogen protecting groups, a valuable result for further synthetic strategies based on the use of lactones of type **2**.

The physical and spectroscopic data of the lactones **2a**–**f** are as follows.

1,4-Oxathiepan-7-one (lactone **2a**): Ketone **1a** (100 mg, 0.862 mmol) was oxidized according to the general procedure to give 55 mg (48%) of **2a** as colorless liquid after *Kugelrohr* distillation; b.p. 200–210°C / 11 mbar (*Kugelrohr* distillation). ^{1}H NMR ($CDCl_3$): 2.76–2.82 (*m*, 2H, H-5), 2.89–2.94 (*m*, 2H, H-3), 3.08–3.14 (*m*, 2H, H-6), 4.52–4.57 (*m*, 2H, H-2); ^{13}C NMR ($CDCl_3$): 24.1 (*t*, C-5), 30.9 (*t*, C-3), 38.8 (*t*, C-6), 71.0 (*t*, C-2), 173.8 (s, C-7); MS for $C_5H_8O_2S$: 132 (M^+, 100%), 102, 74, 60, 46.

1,4-dioxepan-5-one (lactone **2b**): Ketone **1b** (100 mg, 1.000 mmol) was converted according to the general protocol to give 92 mg (79%) of **2b** as colorless liquid after flash column chromatography (silica gel 100:1, petroleum ether: ethyl acetate = 2:1); b.p. 160–165°C / 15 mbar (*Kugelrohr* distillation). ^{1}H NMR ($CDCl_3$): 2.89–2.94 (*m*, 2H, H-6), 3.82–3.87 (*m*, 2H, H-7), 3.89–3.93 (*m*, 2H, H-2), 4.30–4.34 (*m*, 2H, H-3); ^{13}C NMR ($CDCl_3$): 38.9 (*t*, C-6), 64.3 (*t*, C-7), 70.0 (*t*, C-3), 70.4 (*t*, C-2), 173.9 (s, C-5); elemental analysis for $C_5H_8O_3$: calculated: C 51.42, H 6.94, found: C 51.42, H 6.83.

Tetrahydro-4-methyl-1,4-oxazepin-7(2H)-one (lactone **2c**): Ketone **1c** (100 mg, 0.884 mmol) was oxidized according to the general procedure to give 57 mg (50%) of **2c** as colorless liquid after *Kugelrohr* distillation; b.p. 90–95°C/0.01 mbar (*Kugelrohr* distillation). ^{1}H NMR ($CDCl_3$): 2.35 (s, 3H, NCH_3), 2.58–2.64 (*m*, 2H, H-5), 2.70–2.74 (*m*, 2H, H-3), 2.79–2.84 (*m*, 2H, H-6), 4.25–4.29 (*m*, 2H, H-2); ^{13}C NMR ($CDCl_3$): 35.9 (*t*, C-6), 46.1 (q, NCH_3), 51.9 (*t*, C-5), 58.3 (*t*, C-3), 68.3 (*t*, C-2), 174.7 (s, C-7); MS for $C_6H_{11}NO_2$: 129 (M^+), 71, 57, 43 (100%), 42.

Tetrahydro-4-(2-propenyl)-1,4-oxazepin-7(2H)-one (lactone **2d**): Ketone **1d** (100 mg, 0.719 mmol) was converted according to the general protocol and the product mixture obtained was analyzed by GC/MS. Product **2d** was detected in 10% yield. MS for $C_8H_{13}NO_2$: 155 (M^+), 96, 82, 68, 55, 42 (100%), 41.

Tetrahydro-4-acetyl-1,4-oxazepin-7(2H)-one (lactone **2e**): Ketone **1e** (100 mg, 0.709 mmol) was oxidized according to the general procedure in the presence of one equivalent of β-cyclodextrin to give 45 mg (39%) of **2e** and 33 mg of recovered **1e** (59% yield based on recovered starting material). Compound **2e** was isolated as colorless crystals after flash column chromatography (silica gel 100:1, tetrahydrofuran: dichloromethane = 1:1); m.p. 90–93°C. The product showed E/Z isomerism for the acetyl group in all NMR spectra: ^{1}H NMR ($CDCl_3$): 2.13 and 2.15 (2 q, 3H, $COCH_3$, E/Z isomers), 2.76–2.86 (*m*, 2H, H-6), 3.63–3.94 (*m*, 4H, H-3 and H-5), 4.22–4.30 (*m*, 2H, H-2); ^{13}C NMR ($CDCl_3$): 21.3 and 21.6 (2 q, CH_3, E/Z isomers), 36.6 and 37.7 (2*t*, C-6, E/Z isomers), 38.4, 43.4, 45.3, and 50.0 (4*t*, C-3 and C-5, E/Z isomers), 68.7 and 69.3 (2*t*, C-2, E/Z isomers), 169.2 (s, CON), 172.9 (s, C-7); elemental analysis for $C_7H_{11}NO_3$: calculated: C 53.49, H 7.05, N 8.91, found: C 53.24, H 6.98, N 8.62.

Tetrahydro-7-oxo-1,4-oxazepin-4(5H)-carboxylic acid methylester (lactone **2f**): Ketone **1f** (100 mg, 0.639 mmol) was reacted according to the general procedure in the presence of one equivalent of β-cyclodextrin to give 44 mg (40%) of **2f** and 40 mg of recovered **1f** (67% yield based on recovered starting material). Compound **2f** was isolated as colorless oil after flash column chromatography (silica gel 100:1, petroleum ether: ethyl acetate = 3:1); ^{1}H NMR ($CDCl_3$): 2.70–2.76 (*m*, 2H, H-6), 3.60–3.64 (*m*, 2H, H-5), 3.65 (s, 3H, OCH_3), 3.71–3.75 (*m*, 2H, H-3), 4.17–4.21 (*m*, 2H, H-2); ^{13}C NMR ($CDCl_3$): 37.2 (*t*, C-6), 41.0 (*t*, C-5), 47.6 (*t*, C-3), 53.0 (q, OCH_3), 69.0 (*t*, C-2), 155.5 (s, COO), 173.4 (s,

[4] For an excellent review about *N*-acyl protecting groups, see Ref. [29] and references therein.

C-7); elemental analysis for $C_7H_{11}NO_4$: calculated: C 48.55, H 6.40, N 8.09, found: C 48.28, H 6.12, N 7.87.

4. Conclusion

The range of substrates known to be oxidized by CHMO has been extended to include several representative heterocyclic ketones. Whole cells of our recently introduced engineered *E. coli* strain provided a simple means of accomplishing Baeyer-Villiger oxidations without the need for enzyme purification or cofactor regeneration. Moreover, no side reactions of oxidation-sensitive functionalities such as sulfur or nitrogen were observed, demonstrating that both native *E. coli* enzymes and CHMO possess high chemoselectivities. While both electron-donating and withdrawing protecting groups at nitrogen were tolerated by CHMO, membrane penetration may be a limiting factor in some cases.

Acknowledgements

This project was funded by the Oesterreichische Nationalbank (grant no. JF-7619) and the Hochschul-Jubiläumsfond der Stadt Wien (grant no. H-40/98). Support by Baxter Immuno Austria is gratefully acknowledged. The authors also thank Florian Rudroff and Anna Innitzer for preliminary experiments in this area and Prof. Christian Kubicek and Dr. Robert Mach (both Vienna University of Technology, Institute for Biochemical Technology and Microbiology) for their support and many helpful discussions.

References

[1] J.D. Stewart, Curr. Org. Chem. 2 (1998) 211.
[2] P.W. Trudgill (Ed.), Microbial Degradation of the Alicyclic Ring, Structural Relationships and Metabolic Pathways 13, Marcel Dekker, New York, 1984, p. 131.
[3] G. Grogan, S. Roberts, P. Wan, A. Willets, Biotechnol. Lett. 15 (1993) 913.
[4] C. Bolm, G. Schlingloff, K. Weickhardt, Angew. Chem., Int. Ed. Engl. 33 (1994) 1848.
[5] A. Gusso, C. Baccin, F. Pinna, G. Strukul, Organometallics 13 (1994) 3442.
[6] C. Bolm, G. Schlingloff, F. Bienewald, J. Mol. Catal. A: Chem. 117 (1997) 347.
[7] G. Strukul, A. Varagnolo, F. Pinna, J. Mol. Catal. A: Chem. 117 (1997) 413.
[8] C. Bolm, T.K.K. Luong, G. Schlingloff, Synlett (1998) 1151.
[9] N.A. Donoghue, D.B. Norris, P.W. Trudgill, Eur. J. Biochem. 63 (1976) 175.
[10] J.D. Stewart, K.W. Reed, M.M. Kayser, J. Chem. Soc., Perkin Trans. 1 (1996) 755.
[11] J.D. Stewart, K.W. Reed, J. Zhu, G. Chen, M.M. Kayser, J. Org. Chem. 61 (1996) 7652.
[12] J.D. Stewart, K.W. Reed, C.A. Martinez, J. Zhu, G. Chen, M.M. Kayser, J. Am. Chem. Soc. 120 (1998) 3541.
[13] M.M. Kayser, G. Chen, J.D. Stewart, Synlett (1999) 153.
[14] G. Chen, M.M. Kayser, M.D. Mihovilovic, M.E. Mrstik, C.A. Martinez, J.D. Stewart, New J. Chem. 23 (1999) 827.
[15] J.A. Latham, C.T. Walsh, J. Am. Chem. Soc. 109 (1987) 3421.
[16] E.A. Fehnel, M. Carmack, J. Am. Chem. Soc. 70 (1948) 1813.
[17] W.E. Solomons, J. Heterocycl. Chem. 19 (1982) 1035.
[18] L.D. Wise, I.C. Pattison, D.E. Butler, H.A. DeWald, E.P. Lewis, S.J. Lobbestael, H. Tecle, L.L. Coughenour, D.A. Downs, B.P.H. Poschel, J. Med. Chem. 28 (1985) 1811.
[19] M. Shimano, A.I. Meyers, J. Org. Chem. 60 (1995) 7445.
[20] J. Meinwald, J.J. Tufariello, J.J. Hurst, J. Org. Chem. 29 (1964) 2914.
[21] F.W. Studier, B.A. Moffatt, J. Mol. Biol. 189 (1986) 113.
[22] J.C. Craig, K.K. Purushothaman, J. Org. Chem. 35 (1970) 1721.
[23] Y. Ogata, Y. Sawaki, in: W.J. Mijs, C.R.H.I. de Jonge (Eds.), Organic Synthesis by Oxidation with Metal Compounds, Plenum, New York, 1986, p. 839.
[24] J.P. Ferris, R.D. Gerwe, G.R. Gapski, J. Org. Chem. 33 (1968) 3493.
[25] E.E. Smissman, A. Makriyannis, J. Org. Chem. 38 (1973) 1652.
[26] J.H. Cooley, E.J. Evain, Synthesis (1989) 1.
[27] F. Guibe, Tetrahedron 53 (1997) 13509.
[28] F. Guibe, Tetrahedron 54 (1998) 2967.
[29] P.J. Kocienski, Protecting Groups, Georg Thieme Verlag, Stuttgart, 1994, p. 186.
[30] R. Bar, Trends Biotechnol. 7 (1989) 2.