Baeyer–Villiger Monooxygenases, an Emerging Family of Flavin-Dependent Biocatalysts

Nanne M. Kamerbeek,[a] Dick B. Janssen,[a] Willem J. H. van Berkel,[b] Marco W. Fraaije[a,*]

[a] Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
Fax: (+31)-50-3634165, e-mail: kamerbeek@chem.rug.nl, m.w.fraaije@chem.rug.nl, d.b.janssen@chem.rug.nl
[b] Laboratory of Biochemistry, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands
Fax: (+31)-317-484801, e-mail: willem.vanberkel@wur.nl

Received: January 20, 2003; Accepted: February 24, 2003

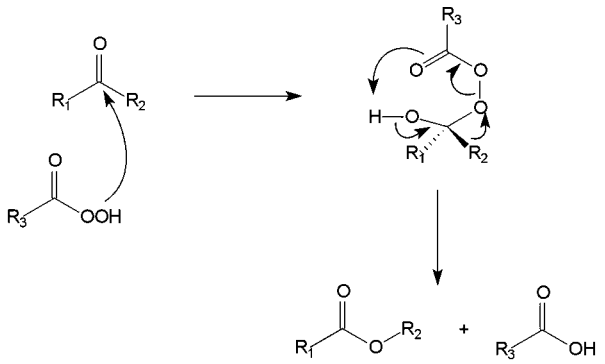
Abstract: Baeyer–Villiger monooxygenases (BVMOs) are flavoenzymes that catalyze a remarkably wide variety of oxidative reactions such as regio- and enantioselective Baeyer–Villiger oxidations and sulfoxidations. Several of these conversions are difficult to achieve using chemical approaches. Due to their selectivity and catalytic efficiency, BVMOs are highly valuable biocatalysts for the synthesis of a broad range of fine chemicals. For a long time, only one member of this class of flavin-containing biocatalysts had been cloned and overexpressed which has limited their application for synthetic processes. Recently a number of new genes that encode BVMOs have been sequenced and overexpressed. In this paper the biocatalytic properties of recently cloned BVMOs are reviewed. Furthermore, the potential for obtaining novel BVMOs from sequenced genomes will be discussed.

Keywords: Baeyer–Villiger oxidation; biocatalysis; enantioselectivity; flavoprotein; monooxygenase; sulfoxidation

1 Introduction

The conversion of ketones into esters or cyclic ketones into lactones was discovered more than a century ago by Adolf von Baeyer and Victor Villiger.[1] In this reaction, the ketone is attacked by a nucleophilic peroxy acid to form the so-called tetrahedral 'Criegee intermediate' (Scheme 1).[2] This unstable species undergoes a rearrangement *via* expulsion of a carboxylate ion and migration of a carbon-carbon bond, yielding the ester and the acid. In general, the most substituted carbon center migrates with retention of configuration. Steric, conformational, and electronic factors have influence on the rate of rearrangement and the migration preferences. Migration is also influenced by the type of peroxy acid used.[3] These features make the Baeyer–Villiger reaction an interesting tool for the synthesis of lactones and esters.

Unfortunately, the general use of peracids like 3-chloroperoxybenzoic acid or peroxotrifluoroacetic acid as reagents and the use of solvents have several



Scheme 1. Mechanism of the Baeyer–Villiger oxidation by peracids.

 DOI: 10.1002/adsc.200303014

Nanne Kamerbeek (born 1973) is doing since 1998 his graduate studies on the Baeyer–Villiger biocatalyst, 4-hydroxyacetophenone monooxygenase, from *Pseudomonas fluorescens* ACB in the Biotechnology group of Prof. Dick Janssen at the University of Groningen.

Dick Janssen (born 1954) did his Ph. D. studies at the University of Nijmegen, investigating nitrogen metabolism in *Pseudonomas*, with Prof. G. D. Vogels. He worked as a postdoctoral and Royal Academy of Sciences fellow at the University of Groningen and is now a professor of biotechnology there. His research interest is the biotransformation of synthetic compounds, with emphasis on the molecular enzymology of dehalogenation reactions, as well as the engineering of enzymes for biocatalysis.

Willem van Berkel (born 1952) did his graduate studies on the structure-function relationship of *p*-hydroxybenzoate hydroxylase at Wageningen University under Professor Franz Mueller. At present, he is an associate professor in biochemistry at Wageningen University. His research interests are in molecular enzymology, with particular emphasis on the structure, function, redesign and application of oxygenases and oxidases.

Marco Fraaije (born 1968) did his graduate studies on vanillyl-alcohol oxidase at Wageningen University under the supervision of Dr. Willem van Berkel. After an EMBO postdoctoral fellowship in protein crystallography at the University of Pavia (Prof. Andrea Mattevi) he moved in 1999 to the Department of Biochemistry of Groningen University. As an assistant professor within the Biotechnology group of Prof. Dick Janssen he is responsible for research on oxidative biocatalysts. Except for developing new biocatalytic tools for the production of fine chemicals, the research aims at a better understanding of sequence-function relationship of redox enzymes.

disadvantages that do not match with the principles of green chemistry.[4] Firstly, due to the shock-sensitivity and explosive character of peracids, large-scale reactions increase the potential risk for accidents. Secondly, the use of halogenated reagents and solvents is environmentally unfriendly. Thirdly, peracids are powerful oxidative agents. Therefore laborious protection and deprotection steps are needed in synthesis in order to prevent by-product formation.

To avoid the use of peracids, transition metal catalysts[5] and organocatalytic compounds[6–8] have been developed which use hydrogen peroxide or oxygen as milder oxidant for the Baeyer–Villiger reaction. An even more 'green' method has recently been applied by Bolm et al. as they used compressed CO_2 as a solvent, establishing Baeyer–Villiger oxidation of various ketones using oxygen as primary oxidant and benzaldehyde or pivalaldehyde as co-reductant.[9] Despite these efforts, the development of biocatalytic processes would be highly attractive for performing enantio- and regioselective Baeyer–Villiger oxidations in an environmentally benign way.

The first example of a biological Baeyer–Villiger reaction dealt with the biotransformation of steroids by fungi and was discovered in 1948.[10] Since then, Baeyer–Villiger oxidation steps have been found in biosynthetic pathways in many different organisms, e.g., aflatoxin synthesis in fungi,[11] synthesis of iridoids and steroids in plants,[12,13] and toxin synthesis in shellfish.[14] Baeyer–Villiger oxidation steps have also been frequently observed in microbial degradation pathways. Microorganisms have been found to use Baeyer–Villiger monooxygenases (BVMOs) in order to grow on aliphatic methyl ketones,[15,16] alicyclic hydrocarbons,[17–19] aromatic compounds,[20–27] and terpenes.[28,29]

All BVMOs characterized to date are NAD(P)H-dependent flavoproteins. They incorporate one atom of molecular oxygen into the substrate and the other atom is reduced to water. BVMOs can be classified in two groups: Type I BVMOs contain flavin adenine dinucleotide (FAD) as cofactor, use NADPH as source for electrons and consist of identical subunits, while Type II BVMOs contain flavin mononucleotide (FMN) as cofactor, use NADH as electron donor and are com-

posed of $\alpha_2\beta$ trimers.[30] So far, all BVMOs that have been cloned could be classified as Type I enzymes while no Type II BVMO sequence is known. Unfortunately, there are no crystal structures available of any BVMO that would disclose the structural features of this class of enzymes.

Since their discovery much research has been performed to explore the biocatalytic properties of BVMOs, either using whole-cells or isolated enzymes. BVMOs are able to catalyze a remarkable wide variety of oxidative reactions such as regio- and enantioselective Baeyer–Villiger oxidations and sulfoxidations, reactions which are difficult, if not impossible, to be achieved using chemical approaches (see reviews[30–35]). However, until a few years ago only one BVMO, cyclohexanone monooxygenase (CHMO; EC 1.14.13.22), had been cloned and overexpressed, which limited application of this type of biocatalyst for synthetic processes.

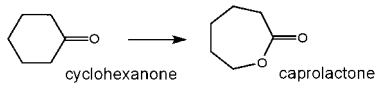
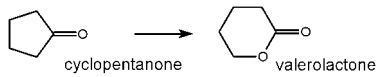
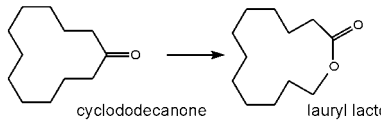
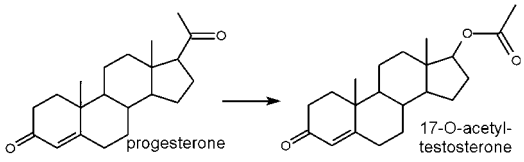
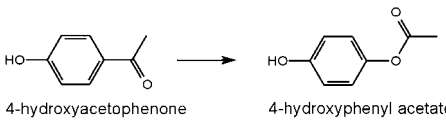
In this review we will focus on the biocatalytic properties of newly identified and characterized Type I BVMOs. Except for the discovery of several CHMO homologues, a number of BVMOs exhibiting novel substrate profiles have been characterized. As all these newly reported BVMOs share sequence homology with CHMO, some recent findings concerning this well-known biocatalyst will be discussed first. Then, we will summarize the biocatalytic properties of cyclopentanone monooxygenase (CPMO; EC 1.14.13.16),[36] cyclododecanone monooxygenase (CDMO; EC 1.14.13.x),[37] steroid monooxygenase (SMO; EC 1.14.13.54),[38] and 4-hydroxyacetophenone monooxygenase (HAPMO; EC 1.14.13.x).[39] Special attention will be given to HAPMO: a BVMO that is primarily active with aromatic compounds. In addition, we will emphasize the potential of novel Type I BVMOs which can be obtained by *in silico* screening of sequenced genomes. Finally, we will discuss other sources for Baeyer–Villiger oxidation biocatalysts.

2 Available Recombinant Baeyer–Villiger Monooxygenases

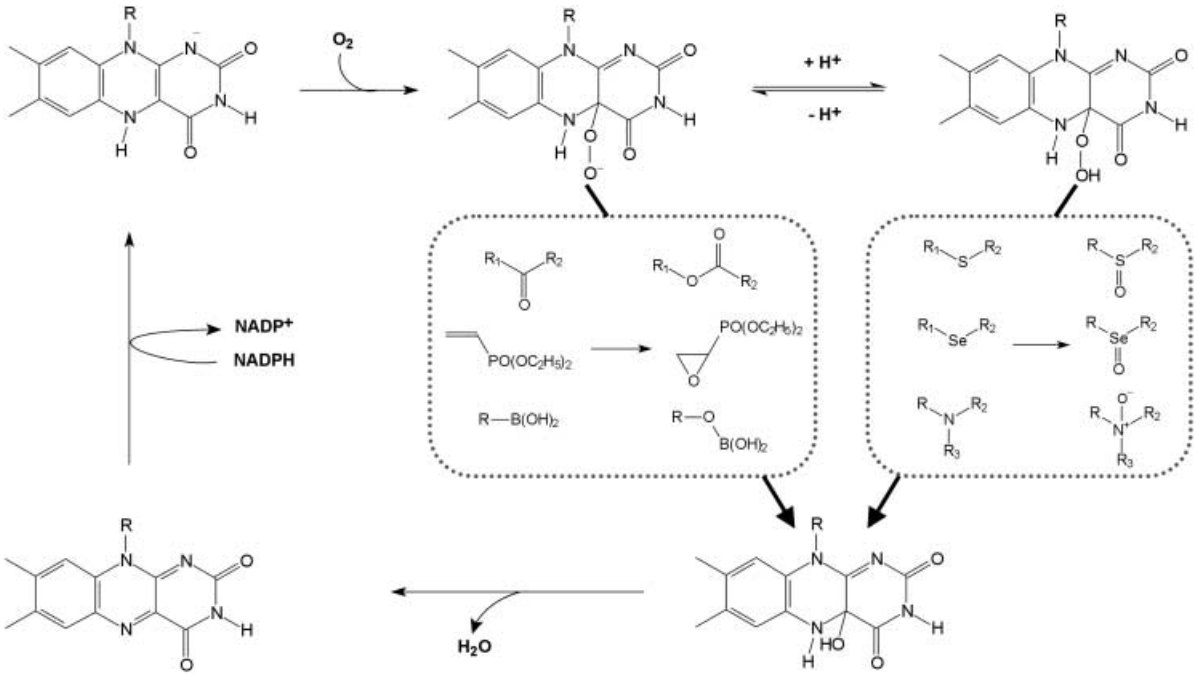
2.1 Cyclohexanone Monooxygenase (CHMO)

CHMO from *Acinetobacter* NCIB 9871 is the most studied Type I BVMO regarding its biocatalytic properties and for a long time this enzyme was the only BVMO of which the gene had been cloned.[40] The CHMO sequence deposited later by Iwaki et al.[41] differed from that originally reported by Chen et al. at several nucleotide positions.[42] Recent mass spectrometry experiments proved that the sequence deposited by Iwaki et al. is the correct CHMO sequence.[43] The CHMO gene encodes a 60.9 kDa protein appearing as monomer upon purification (Table 1). Recently, six other cyclohexanone monooxygenase genes were identified.[44–46] All these newly identified CHMOs show significant protein sequence identity with CHMO from *Acinetobacter* NCIMB 9871. While activity with cyclohexanone

Table 1. Biochemical properties of the characterized Type I BVMOs.

Enzyme *year of cloning*	Catalyzed reaction	Spec. Act. [U/mg][a]	$K_{M,S}$ [μM]	$K_{M,NADPH}$ [μM]	Subunit MW [kDa]	Ref.
CHMO *1988*		30	4	20	60.9	[47]
CPMO *2002*		4.3	< 1	< 3	62.1	[36, 127]
CDMO *2001*		n.d.	n.d.	n.d.	67.5	[37]
SMO *1999*		14	55	0.44	60.1	[38]
HAPMO *2001*		10.5	9.2	64	71.9	[39, 89]

[a] 1 Unit is defined as the amount of enzyme that oxidizes 1 μmol NADPH/min.



Scheme 2. Mechanism of flavin-dependent Baeyer–Villiger monooxygenases mediated oxidation reactions.

has been demonstrated for these novel CHMOs, their biocatalytic potential has not yet been fully explored.

The catalytic mechanism of CHMO has been studied with rapid reaction techniques and proceeds as follows.[47,48] First, the protein-bound FAD is reduced by NADPH, generating the reduced enzyme-$NADP^+$ complex. In the next step, this binary complex reacts with oxygen to form a flavin-peroxide species which undergoes a nucleophilic attack on the carbonyl group of the ketone substrate. The Criegee intermediate thus formed rearranges to the ester product with concomitant formation of a flavin-hydroxide. Finally, water is eliminated from the latter species to reform oxidized FAD and release of $NADP^+$ completes the catalytic cycle (Scheme 2). The ambivalent character of the peroxyflavin is thought to account for the capability of CHMO to catalyze the conversion of both electron-rich and electron-deficient substrates.[31] The electrophilic hydroperoxyflavin catalyzes asymmetric sulfoxidation of various thioethers,[49] oxidation of amines,[50,51] and oxidation of selenides[31] (Scheme 2). The nucleophilic peroxyflavin catalyzes not only Baeyer–Villiger reactions but is also responsible for the observed boron oxidation reactions.[52] The recently reported CHMO-mediated asymmetric epoxidation reactions were also suggested to proceed *via* the nucleophilic peroxyflavin species[53], although it has been proposed that the electrophilic hydroperoxyflavin could catalyze epoxidation reactions.[52]

Numerous experiments, either with (engineered) whole cells or isolated enzyme, have shown that CHMO is a useful biocatalyst for the synthesis of interesting compounds like (bicyclic) lactones,[54] various sulfoxides,[32,49] cyclic sulfates,[55] and thiosulfinates.[56] CHMO displays a remarkably broad substrate specificity allowing conversion of a large variety of ketones and heteroatom-containing compounds. For a number of reactions, the enzyme is highly enantioselective. Recently, it was shown that CHMO can even be used for performing a dynamic kinetic resolution process[57] complying to the need of transformations with (theoretically) 100% yield and 100% ee.[58] In total, more than 100 substrates have been reported for CHMO. A comprehensive overview on reported CHMO-mediated Baeyer–Villiger reactions was recently published by Mihovilovic and coworkers.[35]

Because CHMO is strictly NADPH-dependent, the use of isolated enzyme for large-scale synthesis would require an efficient coenzyme recycling system, as NADPH is too expensive to use in stoichiometric amounts.[59] A well-known NADPH recycling system is the glucose 6-phosphate/glucose 6-phosphate dehydrogenase couple, but the high cost of glucose 6-phosphate is a disadvantage. A cheaper alternative is the formate/formate dehydrogenase system. Two formate dehydrogenases from *Pseudomonas* sp. 110[60] and *Saccharomyces cerevisiae*[61] have been engineered from NAD^+ towards $NADP^+$ preferring enzymes. The *Pseudomonas* enzyme has been used in combination with CHMO-catalyzed conversions.[62,63] Zambianchi et al. tested different NADPH regeneration systems and found optimal results with 2-propanol/alcohol dehydrogenase from *Thermoanaerobium brockii*.[64] An alternative approach for coenzyme recycling is represented by

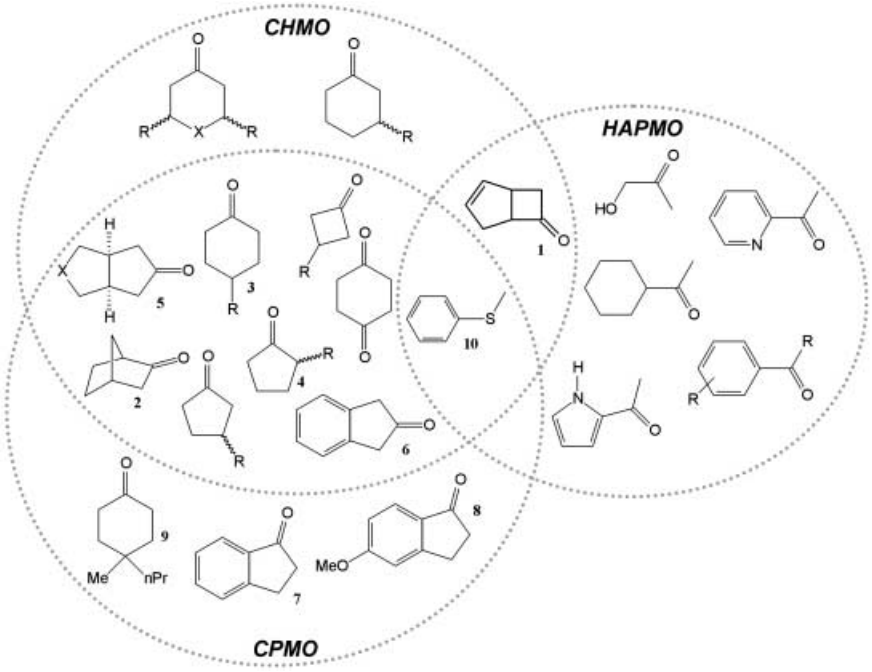


Figure 1. Some representative substrates for cyclohexanone monooxygenase, cyclopentanone monooxygenase and 4-hydroxyacetophenone monooxygenase highlighting the overlapping substrate specificities. Not all the substrates have been tested for every enzyme.

electrochemical regeneration of coenzymes.[65] For example, the pentamethylcyclopentadienyl-rhodium-bipyridine complex $[Cp^*Rh(bpy)(H_2O)]^{2+}$ is reduced by electrons from the cathode and takes up a proton yielding $[Cp^*Rh(bpy)H]^+$ which acts as a hydride transfer reagent on $NAD(P)^+$. This system has successfully been used for synthetic application of a NADH-dependent monooxygenase.[66,67]

Although isolated enzymes have certain advantages, whole cell conversions with oxygenases are attractive for several reasons.[68] One major advantage is the efficient intracellular coenzyme regeneration. Furthermore, in the case of CHMO, heterologous expression circumvents the problem of the lactone hydrolase activity present in the wild-type strain and by this, handling of the pathogenic *Acinetobacter* strain can be avoided. With this in mind, two different *Escherichia coli* strains have been engineered to overexpress CHMO[49,69] and expression in yeast has also been established.[42,70] Whole cell conversions using recombinant CHMO-containing *E. coli* cells has been optimized and applied by Walton and Stewart,[71] who efficiently converted cyclohexanone into ε-caprolactone. Furthermore, Doig et al.,[72] in collaboration with partners from the chemical industry, scaled-up CHMO-mediated whole cell conversion of the ketone bicyclohept-2-en-6-one (Figure 1, compound **1**). The obtained 2-oxabicyclooct-6-en-3-one lactones are valuable intermediates for synthesis of prostaglandins.[73,74]

2.2 Cyclopentanone Monooxygenase (CPMO)

CPMO was purified in 1976 by Griffin et al. from *Comamonas* (previously *Pseudomonas*) sp. NCIMB 9872 growing on cyclopentanone.[18] Last year a gene cluster involved in cyclopentanone catabolism of this microorganism was characterized, and the gene encoding CPMO was cloned and expressed in *E. coli*.[36] In contrast with CHMO, being a monomer, CPMO appears as a tetramer upon purification. The subunit molecular mass is 62.1 kDa (Table 1).

Most biocatalytic studies with CPMO have been performed with whole cells.[75–77] Initial tests showed that the enzyme prefers C_4 to C_8 ketones and norbornanone (**2**) as substrates.[18] These compounds are also oxidized by CHMO indicating that the substrate specificities of these two BVMOs overlap (Figure 1). Although several substrates can be converted by both CPMO and CHMO, the enantioselectivity can differ. For example, the prochiral compound 4-methylcyclohexanone (**3**, R = CH_3) is converted by CHMO to the (*S*)-lactone whereas CPMO produces the (*R*)-lactone.[36] Another example is the conversion of racemic 2-substituted cyclopentanones (**4**). While the CPMO-catalyzed conversions of these cyclopentanones are non-enantioselective,[36] CHMO shows high enantioselectivity with these compounds, enabling kinetic resolution.[78] On the other hand, successful enantioselective oxidation of an unsaturated cyclic ketone, 5-hexyl-2-cyclopentenone and of

2-(2′-acetoxyethyl)cyclohexanones was reported for CPMO.[76] These conversions are of interest because many functionalized chiral δ-valerolactones are biologically active compounds and valuable intermediates in natural product synthesis.[79]

CPMO is also able to catalyze oxidation of the prochiral fused system (**5**) forming the opposite enantiomer to that obtained with CHMO.[35,80] Comparison of CHMO and CPMO also showed that several CPMO substrates are not accepted by CHMO. While the non-conjugated indan-2-one (**6**) is converted by both CHMO and CPMO, indan-1-one (**7**) and 5-methoxyindan-1-one (**8**) could only be converted by CPMO.[36] The same result was recently found by Furstoss and coworkers who tested various substituted 1-indanones with whole cells expressing CHMO, CPMO or HAPMO. Only CPMO-expressing cells converted some of the tested indanones.[81] Also 4-methyl-4-*n*-propylcyclohexanone (**9**) is only a substrate for CPMO.[36] This clearly indicates that the substrate acceptance of CPMO broadens the scope of Baeyer–Villiger reactions that can be performed using recombinant enzymes.

2.3 Cyclododecanone Monooxygenase (CDMO)

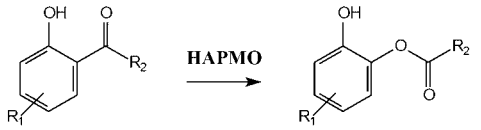
Alicyclic hydrocarbons, like cyclopentane, cyclohexane and cyclododecane, are major components of petroleum. Baeyer–Villiger oxidation is one of the first steps in the microbial degradation of these compounds.[82] In 1999, the first cyclododecanone monooxygenase (CDMO) from *Rhodococcus ruber* CD4 was purified and characterized.[17] From another strain, *Rhodococcus ruber* SC1, a gene cluster involved in cyclododecanone oxidation has been identified and the gene encoding CDMO was cloned and expressed in *E. coli*.[37] From the protein sequence it can be deduced that CDMO from *R. ruber* SC1 is a 67.5 kDa protein (Table 1). Expression of CDMO enabled the use of whole cells for CDMO-mediated bioconversions. These experiments showed that CDMO efficiently converts C_{11} – C_{15} cyclic ketones. Insignificant conversion was observed with C_6 and C_{10} cyclic ketones and the enzyme was inactive towards C_7 and C_8 cyclic ketones.[37] By accepting bulky aliphatic cyclic ketones, compounds that are not accepted by CPMO and CHMO, this novel BVMO can be of great biocatalytic value. For example, CDMO is a suitable biocatalyst to produce lauryl lactone (Table 1), a compound for which a chemical synthesis route is not known.[83]

2.4 Steroid Monooxygenase (SMO)

As mentioned in the introduction, the first discovered BVMO activities involved microbial conversion of steroids[10]. Two steroid monooxygenases performing Baeyer–Villiger reactions have been purified and characterized.[84,85] These SMOs from *Cylindrocarpon radicicola* and *Rhodococcus rhodochrous* have different substrate specificities. Whereas the *Rhodococcus* enzyme only catalyzes the esterification of the progesterone side-chain towards testosterone acetate (Table 1),[84] the fungal enzyme also catalyzes oxidative lactonization of androstenedione to testololactone.[84] A recombinant expression system for *R. rhodochrous* SMO has been constructed in *E. coli*.[38] This resulted in a 40-fold higher protein production compared to the level in *R. rhodochrous*. Some biochemical characteristics of SMO are listed in Table 1. Because no further biocatalytic studies have been reported for this BVMO, it would be worthwhile to test other substrates. While SMO has evolved to catalyze oxidations of steroids, it might also be able to convert compounds unrelated to its physiological substrate as has been observed for CHMO. Such promiscuity in substrate specificity has also been found for another flavoprotein acting on steroids: cholesterol oxidase from *Rhodococcus erythropolis*. This FAD-containing oxidase was found to be able to perform enantioselective oxidations of a range of secondary alcohols.[86]

2.5 4-Hydroxyacetophenone Monooxygenase (HAPMO)

Microbial Baeyer–Villiger oxidation of aromatic compounds was first reported in the mid 1970's.[22] Several of these aromatic degradation pathways involving Baeyer–Villiger oxidation have been elucidated which include the catabolic routes for acetophenones,[21–24] 1-phenylethanol,[23] 4-ethylphenol,[20] and fluorene.[25] The first purification of a BVMO active on aromatic compounds was only described in 1999.[87] This enzyme, 4-hydroxyacetophenone monooxygenase (HAPMO), was isolated from *Pseudomonas fluorescens* ACB[24] growing on 4-hydroxyacetophenone (Table 1). Recently, also a HAPMO homologue from *Pseudomonas putida* JD1, an organism growing on 4-ethylphenol which is degraded *via* 4-hydroxyacetophenone, has been purified and characterized.[88] HAPMO from *P. fluorescens* ACB is a homodimer of 145 kDa with each subunit containing a tightly non-covalently bound FAD. In *P. fluorescens* ACB, the enzyme oxidizes 4-hydroxyacetophenone to 4-hydroxyphenyl acetate. The enzyme has a strong preference for NADPH over NADH, as has been found for all BVMOs described above, and is optimally active around pH 8. In 2001, the HAPMO encoding gene (*hapE*) was cloned which allowed overexpression of the recombinant biocatalyst in *E. coli*.[39] The *hapE* gene is the fifth gene in an operon encoding the genes involved in the degradation of 4-hydroxyacetophenone in *P. fluorescens* ACB. The fourth gene (*hapD*) encodes the 4-hydroxyphenyl acetate hydrolase.[39]



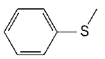
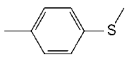
Scheme 3. Biocatalytic production of protected catechols using HAPMO.

Initially, it was found that the substrate specificity of HAPMO covers a wide range of aryl ketones with a preference for compounds bearing an electron-donating substituent at the *para*-position of the aromatic ring.[39] Further studies revealed that HAPMO is also capable of catalyzing the Baeyer–Villiger oxidation of a wide variety of other ketones including several heteroaromatic and aliphatic compounds (Figure 1), while also some sulfides were shown to be readily converted.[89] Being the first recombinant BVMO that acts primarily on aromatic compounds, HAPMO represents a promising biocatalytic tool as will be exemplified below.

Chemical synthesis of a phenol- or catechol-containing compound often requires protection of the hydroxy group(s) to prevent oxidation reactions. For this, ethers are the most widely used protective groups, while esterification is an important alternative.[90] The HAPMO-catalyzed conversion of ring-substituted aryl ketones into their corresponding phenyl acetates provides a biocatalytic alternative for the synthesis of protected phenols and partially protected catechols (Scheme 3).[91] Substituted catechols are valuable precursors for the synthesis of pharmaceutical compounds.[92–94] Synthesis of these compounds requires the use of purified enzyme because with whole cells, the presence of a highly active esterase in *P. fluorescens* ACB prevents the accumulation of the desired products. Alternatively, *E. coli* cells that overexpress HAPMO can be used. ^{19}F NMR studies showed that the rate of the HAPMO-mediated conversion of 4-fluoracetophenones is optimal at pH 8 but that the fluorophenyl acetates are better stabilized at pH 6.[91]

HAPMO is also able to perform a Baeyer–Villiger oxidation of 4-hydroxybenzaldehyde. Only the ester is formed indicating that, as observed for the conversion of aryl ketones, the aromatic ring is the migrating group. Enzymatic or chemical hydrolysis of the substituted phenyl acetates formed by HAPMO oxidation of aryl ketones or benzaldehydes gives access to substituted phenols and dihydroxybenzenes. This biocatalytic route can be exploited for producing ^{18}F-fluorophenols from the corresponding ^{18}F-labeled aldehyde or ketone precursors. These fluorinated phenols find applications as radiotracers for positron emission tomography (PET).[95] Substituted phenols obtained by chemical Baeyer–Villiger oxidation of aromatic aldehydes can also be used as building blocks for the synthesis of coumestans, which are biologically active flavonoids.[96] Furthermore, hydrolysis of 4-hydroxyphenyl acetate affords hydroquinone which is, like other dihydroxybenzenes, an important intermediate of organic synthesis.[97] Because chemical methods to synthesize hydroquinone use volatile and carcinogenic benzene as starting material, there is a growing interest in alternative routes to produce hydroquinone.[98]

Table 2. Enantiomeric excess of sulfoxides produced by different BVMOs.

Substrate	HAPMO[89]	CHMO[128]	CPMO[a],[77]
 methyl phenyl sulfide	>99% (*S*)	99% (*R*)	100% (*S*)
 methyl *p*-tolyl sulfide	>99% (*S*)	37% (*S*)	84% (*S*)

[a] Experiments performed with whole cells.

It has also been found that HAPMO can be used to produce chiral sulfoxides. Enantiomerically pure sulfoxides are of high interest for synthetic chemists as they influence the stereoselectivity of reactions at nearby centers.[99] HAPMO performs highly enantioselective oxidation of methyl phenyl sulfide (**10**) and methyl *p*-tolyl sulfide.[89] Interestingly, compared to CHMO and CPMO, HAPMO shows a better performance in methyl *p*-tolyl sulfide oxidation (Table 2).

In contrast to CHMO,[74] HAPMO displays no regioselectivity during conversion of racemic bicyclohept-2-en-6-one (**1**). Low ee values have been obtained for the different lactones. On the other hand, HAPMO prefers (1*R*,5*S*) bicyclohept-2-en-6-one above the (1*S*,5*R*) enantiomer exhibiting an *E*-value of 20. Therefore, HAPMO can be used for a kinetic resolution to obtain the (1*S*,5*R*) enantiomer.[89]

As discussed above for CHMO, several strategies of cofactor regeneration can be employed. In biocatalytic applications using isolated enzymes, recycling is commonly achieved by using a dehydrogenase. Unfortunately, most of these dehydrogenases are only active with NADH. Clearly, a BVMO that would accept NADH as electron donor would be highly interesting for isolated enzyme applications. Furthermore, even though whole cells can be used to circumvent the need of a coenzyme recycling system, the rate of cellular NADPH recycling might still limit efficient catalysis. A change in specificity towards NADH could also be beneficial for biocatalytic applications using whole cells as NADH levels in the cell are relatively high.[100,101] For several flavoproteins a switch in coenzyme specificity has been achieved by enzyme engineering[102–104] but no Baeyer–Villiger monooxygenase has been engineered for this purpose. Recently, we have identified several amino acid residues of HAPMO that are involved in the

recognition of the 2′-phosphate moiety of NADPH. By changing one of these residues a shift in coenzyme preference toward NADH could be established. (manuscript in preparation,[105]) However, the observed NADH affinity of the engineered HAPMO variant is still not satisfactory. Clearly, more residues need to be changed to improve the NADH specificity in terms of catalytic efficiency. For engineering a more effective NADH-specific BVMO, a crystal structure of HAPMO or any other homologous BVMO would be desirable.

3 Genome Harvesting of Novel Baeyer–Villiger Monooxygenases

As described above, the recent effort to discover new Baeyer–Villiger biocatalysts has resulted in the identification and sequencing of a number of BVMO genes. Without exception, all novel BVMOs could be classified as Type I BVMOs based on their biochemical properties (intracellular, soluble, FAD-containing, NADPH-dependent, specific activities around 10 U mg^{-1}, subunits of typically ~60 kDa) (Table 1). In addition, the sequence information of these novel BVMOs allowed a sequence comparison study of Type I BVMOs.[106] This revealed that Type I BVMOs are part of a superfamily of sequence-related flavin-dependent monooxygenases. Enzymes belonging to this flavoprotein superfamily typically contain two dinucleotide binding sequence motifs (GxGxxG/A) which are involved in binding of the cofactor FAD and the coenzyme NAD(P)H. All characterized members of this flavoprotein superfamily have indeed been shown to be dependent on FAD and require NAD(P)H as electron donor. Based on sequence homology, three monooxygenase subfamilies can be recognized within this novel superfamily: (1) the so-called flavin-monooxygenase (FMO) family mainly consisting of heteroatom-oxidizing monooxygenases from eukaryotic origin, (2) a family of bacterial amine-hydroxylating monooxygenases (NMOs), and (3) a family of Type I BVMOs. Interestingly, sequences belonging to the BVMO subfamily could specifically be recognized by a strictly conserved sequence motif (FxGxxxHxxxW(P/D)) which is not present in members from the other two subfamilies.[106] A site-directed mutagenesis study of HAPMO showed that residues conserved within this sequence motif are critically involved in catalysis. Replacing the strictly conserved histidine in HAPMO by an alanine resulted in an inactive protein while mutagenesis of the conserved tryptophan resulted in impaired protein folding.[106]

Except for the identification of residues that are of importance to catalyze a Baeyer–Villiger reaction, the BVMO-specific fingerprint sequence also helps to identify new BVMO sequences and thereby allows efficient harvesting of novel biocatalysts from sequenced genomes. For example, genome sequences can be searched for the presence of Type I BVMO sequences. By performing a pattern-hit search *via* the PEDANT database (http://pedant.gsf.de), each available genome can be probed for the occurrence of BVMO genes (Table 3). Using this search tool we have found that Type I BVMO genes are present in approximately 15% of all sequenced microbial genomes. The commonly used hosts for protein expression, *E. coli* and *S. cerevisiae*, do not contain any Type I BVMO gene. This confirms the suitability of these microbial hosts for recombinant BVMO production as they exhibit no competing BVMO activity. BVMO genes are frequently found in genomes from pathogenic bacteria including *Mycobacterium tuberculosis* which contains 6 putative BVMO sequences. One of these putative BVMOs has recently been reported to be a FAD-containing monooxygenase that is responsible for activation of antitubercular drugs by catalyzing a sulfoxidation reaction.[107] We have shown that this enzyme is also able to perform Baeyer–Villiger oxidations, suggesting that it indeed catalyzes a Baeyer-Villiger reaction *in vivo* (Fraaije et al., manuscript in preparation). This is in line with the observation that Mycobacteria catalyze a variety of Baeyer–Villiger reactions.[108,109] Occurrence of BVMOs is not restricted to bacteria as also some BVMO sequences could be identified in genomes from eukaryotic microorganisms like *Aspergillus parasiticus*. In line with this, a Baeyer–Villiger oxidation step has been observed in the biosynthesis of aflatoxin by this fungus.[110] So far, no Type I BVMO has been found in genome sequences from Archaea. This can be explained by the fact that Archaea live in extreme environments were oxygen is often not available. Furthermore, no BVMO could be identified in the available plant genomes or the human genome.

To perform a sequence motif based trawl of multiple genomes, the BVMO-identifying sequence motif can be used for a pattern-hit initiated (PHI) BLAST search.[111] With such an approach, protein sequences are retrieved that share sequence similarity with known BVMO sequences while they also contain the sequence motif. The latter effectively prevents false hits excluding members of the FMO or NMO families. A PHI-BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) per-

Table 3. Occurrence of Type I BVMOs in some selected microbial genomes.

Organism	Total number of genes	Number of Type I BVMO genes
E. coli	4289	0
M. tuberculosis H37Rv	3924	6
M. leprae TN	1605	1
S. coelicolor A3(2)	7512	2
P. aeruginosa PAO1	5565	3
S. cerevisiae	6449	0

formed on December 2nd 2002 yielded a set of 68 putative microbial BVMO sequences. This set of sequences, including all cloned BVMOs described above, mainly consisted of uncharacterized putative BVMO genes. This indicates that a large pool of unexplored BVMOs is available for biocatalytic exploration.

4 Alternative Baeyer–Villiger Biocatalysts

Except for Type I and Type II BVMOs, a small number of other enzymes has been shown to catalyze Baeyer–Villiger reactions. One example is the conversion of aromatic aldehydes into the corresponding formate esters by a pig liver enzyme.[112] This enzyme was found to belong to the above-mentioned FMO family and therefore is related to Type I BVMOs.[106] As described above, these FMOs share several properties with Type I BVMOs. They contain FAD as redox cofactor, use NADPH for activity, and are able to catalyze a multitude of oxygenation reactions. Similar to BVMOs, FMOs use the oxygenated flavin as the reactive species during catalysis. In mammals FMOs are primarily involved in converting amines and other heteroatom-containing compounds and serve a role in detoxification similar to cytochrome P450.[113] While Baeyer–Villiger reactions have only been observed for an FMO from pig liver, other FMOs might also be capable of catalyzing Baeyer–Villiger reactions. A genome search using a FMO specific fingerprint sequence[106] revealed that FMO genes are abundant in eukaryotic genomes while their number is low in bacterial genomes. Plants contain a relatively large number of FMOs and therefore might represent a promising source for interesting oxygenating biocatalysts. Interestingly, Baeyer–Villiger oxidation steps have been noted in plants[12,13] but it is unknown by which enzyme(s) these reactions are catalyzed.

Another enzyme that has been shown to catalyze Baeyer–Villiger reactions is the NADH- and FMN-dependent luciferase from the light-emitting bacterium *Vibrio fischeri*. This enzyme catalyzes the *in vivo* oxidation of aldehydes into their acids with the concomitant production of light. The mechanism for this oxidation reaction was proposed to be similar to a Baeyer–Villiger reaction, with formation of a peroxyhemiacetal upon the attack of the peroxyflavin on the substrate.[114] Instead of the Baeyer–Villiger rearrangement, a chemically initiated electron luminescence (CIEEL) mechanism has been proposed.[114] Nonetheless, the enzyme is able to convert bicycloheptenone *via* a Baeyer–Villiger type of reaction.[115] Being dependent on FMN and NADH *Vibrio fischeri* luciferase resembles the few characterized Type II BVMOs. Whether these BVMOs are related to luciferase will be clarified as soon as the first Type II BVMO gene sequence is obtained.

Baeyer–Villiger steps have also been reported in the synthesis of polyketides in *Streptomyces* species.[116,117] Recently, a gene from *Streptomyces* has been identified to catalyze the oxidative cleavage of the premithramycin B at the expense of NADPH and oxygen to form the antitumor drug mithramycin. This ring cleavage reaction is thought to proceed *via* a Baeyer–Villiger mechanism suggesting that the enzyme involved is a BVMO. Interestingly, the sequence of the respective enzyme does not contain the Type I BVMO motif and does not share significant sequence similarity with any known Type I BVMO. However, it displays some similarity with members of another flavoprotein family consisting of FAD-containing hydroxylases.[118,119] Members of this family of monooxygenases also rely on the formation of a peroxyflavin intermediate for performing monooxygenation reactions. A major difference with Type I BVMOs is the different way of binding their NAD(P)H coenzyme.[120,121] Another characterized BVMO that cannot be classified as a Type I or Type II enzyme is the cyclohexanone monooxygenase isolated from a *Xanthobacter* sp. as it is FMN- and NADPH-dependent.[122] These examples hint to the existence of classes of BVMOs that do not fall into the groups of Type I or Type II BVMOs.

5 Conclusions and Outlook

BVMOs are extremely useful enzymes for the environmentally friendly synthesis of esters and lactones. The recent characterization of several new BVMOs has expanded the range of enzymatic Baeyer–Villiger reactions. Among these enzymes, HAPMO is particularly useful for the conversion of aromatic ketones and sulfides, allowing the synthesis of a wide range of phenyl acetates or substituted phenols and enantiomerically pure sulfoxides. The recently identified BVMO-specific sequence motif is a powerful tool to find new BVMOs in the rapidly growing collection of sequenced genomes. With the discovery of new BVMOs, new substrates will appear. Combined with the rapid development in technologies like high-throughput screening, construction of recombinant production organisms and fermentation technology the development of new Baeyer–Villiger biocatalysts will be greatly facilitated in the near future.[123]

Unfortunately, no BVMO three-dimensional structure is known to date, preventing a knowledge-based site-directed mutagenesis approach to re-engineer the biocatalytic properties of a specific BVMO. Nonetheless, successful application of random mutagenesis techniques has already been reported for a flavoprotein monooxygenase for which structural data are lacking [124,125]. Therefore, the availability of sequence-related BVMO genes will allow fine-tuning of catalytic properties by exploiting random mutagenesis and gene shuf-

fling methods. Success of such a random enzyme engineering approach relies on efficient screening of mutant libraries. For this, a recently reported BVMO specific activity assay can be used.[126] Taken together, the above-mentioned recent developments will enable strategies to design tailor-made Baeyer–Villiger biocatalysts in the near future.

Acknowledgements

This work was supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO), division 'Procesvernieuwing voor een Schoner Milieu'.

References

[1] A. Baeyer, V. Villiger, *Ber. Dtsch. Chem. Ges.* **1899**, *32,* 3625 – 3633.
[2] R. Criegee, *Justus Liebigs Ann. Chem.* **1948**, *560,* 127 – 135.
[3] M. Renz, B. Meunier, *Eur. J. Org. Chem.* **1999**, 737 – 750.
[4] P. T. Anastas, L. B. Bartlett, M. M. Kirchhoff, T. C. Williamson, *Catalysis Today* **2000**, *55,* 11 – 22.
[5] G. Strukul, *Angew. Chem. Int. Ed.* **1998**, *37,* 1198 – 1209.
[6] S.-I. Murahashi, S. Ono, Y. Imada, *Angew. Chem. Int. Ed.* **2002**, *41,* 2366 – 2368.
[7] C. Mazzini, J. Lebreton, R. Furstoss, *J. Org. Chem.* **1996**, *61,* 8 – 9.
[8] O. Fukuda, S. Sakaguchi, Y. Ishii, *Tetrahedron Lett.* **2001**, *42,* 3479 – 3481.
[9] C. Bolm, C. Palazzi, G. Francio, W. Leitner, *Chem. Commun.* **2002**, 1588 – 1589.
[10] G. E. Turfitt, *Biochem. J.* **1948**, *42,* 376 – 383.
[11] C. A. Townsend, S. B. Christensen, S. G. Davis, *J. Am. Chem. Soc.* **1982**, *104,* 6154 – 6155.
[12] S. Damtoft, H. Franzyk, S. R. Jensen, *Phytochemistry* **1995**, *40,* 773 – 784.
[13] J. Winter, B. Schneider, S. Meyenburg, D. Strack, G. Adam, *Phytochemistry* **1999**, *51,* 237 – 242.
[14] J. L. C. Wright, T. Hu, J. L. McLachlan, J. Needham, J. A. Walter, *J. Am. Chem. Soc.* **1996**, *118,* 8757 – 8758.
[15] F. W. Forney, A. J. Markovetz, *Biochem. Biophys. Res. Commun.* **1969**, *37,* 31 – 38.
[16] L. N. Britton, A. J. Markovetz, *J. Biol. Chem.* **1977**, *252,* 8561 – 8566.
[17] J. D. Schumacher, R. M. Fakoussa, *Appl. Microbiol. and Biotechnol.* **1999**, *52,* 85 – 90.
[18] M. Griffin, P. W. Trudgill, *Eur. J. Biochem.* **1976**, *63,* 199 – 209.
[19] N. A. Donoghue, P. W. Trudgill, *Eur. J. Biochem.* **1975**, *60,* 1 – 7.
[20] K. H. Jones, P. W. Trudgill, D. J. Hopper, *Appl. Environ. Microbiol.* **1994**, *60,* 1978 – 1983.
[21] J. Havel, W. Reineke, *Appl. Environ. Microbiol.* **1993**, *59,* 2706 – 2712.
[22] R. E. Cripps, *Biochem. J.* **1975**, *152,* 233 – 241.
[23] R. E. Cripps, P. W. Trudgill, J. G. Whateley, *Eur. J. Biochem.* **1978**, *86,* 175 – 186.
[24] F. K. Higson, D. D. Focht, *Appl. Environ. Microbiol.* **1990**, *56,* 3678 – 3685.
[25] M. Casellas, M. Grifoll, J. M. Bayona, A. M. Solanas, *Appl. Environ. Microbiol.* **1997**, *63,* 819 – 826.
[26] J. M. Darby, D. G. Taylor, D. J. Hopper, *J. Gen. Microbiol.* **1987**, *133,* 2137 – 2146.
[27] D. J. Hopper, H. G. Jones, E. A., M. E. Rhodes-Roberts, *J. Gen. Microbiol.* **1985**, *131,* 1807 – 1814.
[28] H. J. Ougham, D. G. Taylor, P. W. Trudgill, *J. Bacteriol.* **1983**, 153, 140 – 152.
[29] M. J. van der Werf, A. M. Boot, *Microbiology* **2000**, *5,* 1129 – 1141.
[30] A. Willetts, *Trends Biotechnol.* **1997**, *15,* 55 – 62.
[31] C. T. Walsh, Y.-C. Chen, *Angew. Chem. Int. Ed.* **1988**, *27,* 333 – 343.
[32] S. Colonna, N. Gaggero, P. Pasta, G. Ottolina, *Chem. Commun.* **1996**, *20,* 2303 – 2307.
[33] S. M. Roberts, P. W. H. Wan, *J. Mol Cat. B: Enzym.* **1998**, *4,* 111 – 136.
[34] S. Flitsch, G. Grogan, in *Enzyme Catalysis in Organic Synthesis 2nd edn.,* (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**.
[35] M. D. Mihovilovic, B. Muller, P. Stanetty, *Eur. J. Org. Chem.* **2002**, 3711 – 3730.
[36] N. Iwaki, Y. Hasegawa, S. Wang, M. M. Kayser, P. C. K. Lau, *Appl. Environ. Microbiol.* **2002**, *68,* 5671 – 5684.
[37] K. Kostichka, S. M. Thomas, K. J. Gibson, V. Nagarajan, Q. Cheng, *J. Bacteriol.* **2001**, *183,* 6478 – 6486.
[38] S. Morii, S. Sawamoto, Y. Yamauchi, M. Miyamoto, M. Iwami, E. Itagaki, *J. Biochem.* **1999**, *126,* 624 – 631.
[39] N. M. Kamerbeek, M. J. Moonen, J. G. Van Der Ven, W. J. H. Van Berkel, M. W. Fraaije, D. B. Janssen, *Eur. J. Biochem.* **2001**, *268,* 2547 – 2557.
[40] Y. C. Chen, O. P. Peoples, C. T. Walsh, *J. Bacteriol.* **1988**, *170,* 781 – 789.
[41] H. Iwaki, Y. Hasegawa, M. Teraoka, T. Tokuyama, H. Bergeron, P. C. K. Lau, *Appl. Environ. Microbiol.* **1999**, *65,* 5158 – 5162.
[42] M. J. Cheesman, M. B. Kneller, E. J. Kelly, S. J. Thompson, C. K. Yeung, D. L. Eaton, A. E. Rettie, *Protein Expr. Purif.* **2001**, *21,* 81 – 86.
[43] M. B. Kneller, M. J. Cheesman, A. E. Rettie, *Biochem. Biophys. Res. Commun.* **2001**, *282,* 899 – 903.
[44] Q. Cheng, S. M. Thomas, K. Kostichka, J. R. Valentine, V. Nagarajan, *J. Bacteriol.* **2000**, *182,* 4744 – 4751.
[45] P. C. Brzostowicz, K. L. Gibson, S. M. Thomas, M. S. Blasko, P. E. Rouviere, *J. Bacteriol.* **2000**, *182,* 4241 – 4248.
[46] P. C. Brzostowicz, D. M. Walters, S. M. Thomas, V. Nagarajan, P. E. Rouviere, *Appl. Environ. Microbiol.* **2003**, *69,* 334 – 342.
[47] C. C. Ryerson, D. P. Ballou, C. Walsh, *Biochemistry* **1982**, *21,* 2644 – 2655.
[48] D. Sheng, D. P. Ballou, V. Massey, *Biochemistry* **2001**, *40,* 11156 – 11167.

[49] G. Chen, M. M. Kayser, M. D. Mihovilovic, M. E. Mrstik, C. A. Martinez, J. D. Stewart, *New J. Chem.* **1999**, *23,* 827 – 832.
[50] G. Ottolina, S. Bianchi, B. Belloni, G. Carrea, B. Danieli, *Tetrahedron Lett.* **1999**, *40,* 8483 – 8486.
[51] S. Colonna, V. Pironti, P. Pasta, F. Zambianchi, *Tetrahedron Lett.* **2003**, *44,* 869 – 871.
[52] B. P. Branchaud, C. T. Walsh, *J. Am. Chem. Soc.* **1985**, *107,* 2153 – 2161.
[53] S. Colonna, N. Gaggero, G. Carrea, G. Ottolina, P. Pasta, F. Zambianchi, *Tetrahedron Lett.* **2002**, *43,* 1797 – 1799.
[54] J. D. Stewart, *Curr. Org. Chem.* **1998**, *2,* 195 – 216.
[55] S. Colonna, N. Gaggero, G. Carrea, P. Pasta, *Chem. Commun.* **1998**, 415 – 416.
[56] S. Colonna, N. Gaggero, G. Carrea, P. Pasta, V. Alphand, R. Furstoss, *Chirality* **2001**, *13,* 40 – 42.
[57] N. Berezina, V. Alphand, R. Furstoss, *Tetrahedron Asymmetry* **2002**, *13,* 1953 – 1955.
[58] U. T. Strauss, U. Felfer, K. Faber, *Tetrahedron Asymmetry* **1999**, *10,* 109 – 117.
[59] W. Hummel, *Trends Biotechnol.* **1999**, *17,* 487 – 492.
[60] V. I. Tishkov, A. G. Galkin, G. N. Marchenko, Y. D. Tsygankov, A. M. Egorov, *Biotechnol. Appl. Biochem.* **1993**, *18,* 201 – 207.
[61] A. E. Serov, A. S. Popova, V. V. Fedorchuk, V. I. Tishkov, *Biochem. J.* **2002**, *367,* 841 – 847.
[62] S. Rissom, U. Schwarz-Linek, M. Vogel, V. I. Tishkov, U. Kragl, *Tetrahedron Asymmetry* **1997**, *8,* 2523 – 2526.
[63] U. Schwarz-Linek, A. Krodel, F.-A. Ludwig, A. Schulze, S. Rissom, U. Kragl, V. I. Tishkov, M. Vogel, *Synthesis* **2001**, *6,* 947 – 951.
[64] F. Zambianchi, P. Pasta, G. Carrea, S. Colonna, N. Gaggero, J. M. Woodley, *Biotechnol. Bioeng.* **2002**, *78,* 488 – 495.
[65] W. A. C. Somers, W. van Hartingsveldt, E. C. A. Stigter, J. P. van der Lugt, *Trends Biotech.* **1997**, *15,* 495 – 500.
[66] F. Hollmann, A. Schmid, E. Steckhan, *Angew. Chem. Int. Ed.* **2001**, *40,* 169 – 171.
[67] F. Hollmann, B. Witholt, A. Schmid, *J. Mol. Cat. B: Enzym.* **2002**, *19 – 20,* 167 – 176.
[68] W. A. Duetz, J. B. van Beilen, B. Witholt, *Curr. Opin. Biotechnol.* **2001**, *12,* 419 – 425.
[69] S. D. Doig, L. M. O'Sullivan, S. Patel, J. M. Ward, J. M. Woodley, *Enz. Microbiol. Techn.* **2001**, *28,* 265 – 274.
[70] J. D. Stewart, K. Reed, M. M. Kayser, *J. Chem. Soc. Perkin Trans. 1* **1996**, *8,* 755 – 757.
[71] A. Z. Walton, J. D. Stewart, *Biotechnol. Prog.* **2002**, *18,* 262 – 268.
[72] S. D. Doig, P. J. Avenell, P. A. Bird, P. Gallati, K. S. Lander, G. J. Lye, R. Wohlgemuth, J. M. Woodley, *Biotechnol. Prog.* **2002**, *18,* 1039 – 1046.
[73] R. F. Newton, S. M. Roberts, *Tetrahedron* **1980**, *36,* 2163 – 2196.
[74] V. Alphand, A. Archelas, R. Furstoss, *Tetrahedron Lett.* **1989**, *30,* 3663 – 3664.
[75] H. Sandey, A. Willetts, *Biotechnol. Lett.* **1989**, *11,* 615 – 620.
[76] M. T. Bes, F. Villa, S. M. Robert, P. W. H. Wan, A. Willetts, *J. Mol. Catal. B: Enzym.* **1996**, *1,* 127 – 134.
[77] D. R. Kelly, C. J. Knowles, J. G. Mahdi, I. N. Taylor, M. A. Wright, *Tetrahedron Asymmetry* **1996**, *7,* 365 – 368.
[78] M. M. Kayser, G. Chen, J. D. Stewart, *J. Org. Chem.* **1998**, *63,* 7103 – 7106.
[79] P. Coutrot, C. Grison, C. Bomont, *J. Organometal. Chem.* **1999**, *586,* 208 – 217.
[80] M. D. Mihovilovic, B. Mueller, M. M. Kayser, P. Stane, *Synth. Lett.* **2002**, 700 – 702.
[81] M. C. Gutierrez, V. Alphand, R. Furstoss, *J. Mol. Cat. B: Enzym.* **2003**, *21,* 231 – 238.
[82] Q. Cheng, S. M. Thomas, P. Rouviere, *Appl. Microbiol. Biotechnol.* **2002**, *58,* 704 – 711.
[83] S. M. Thomas, R. DiCosimo, V. Nagarajan, *Trends Biotechnol.* **2002**, *20,* 238 – 242.
[84] E. Itagaki, *J. Biochem.* **1986**, *99,* 815 – 824.
[85] M. Miyamoto, J. Matsumoto, T. Iwaya, E. Itagaki, *Biochim. Biophys. Acta* **1995**, *1251,* 115 – 124.
[86] J.-F. Biellmann, *Chirality* **2002**, *13,* 34 – 39.
[87] M. J. H. Moonen, I. M. C. M. Rietjens, W. J. H. van - Berkel, in *Flavins and Flavoproteins 1999,* (Eds.: S. Ghisla, P. Kroneck, P. Macheroux, H. Sund), Agency for Scientific Publication, Berlin, **1999**.
[88] A. Tanner, D. J. Hopper, *J. Bacteriol.* **2000**, *182,* 6565 – 6569.
[89] N. M. Kamerbeek, A. J. J. Olsthoorn, M. W. Fraaije, D. B. Janssen, *Appl. Environ. Microbiol.* **2003**, *69,* 419 – 426.
[90] T. W. Greene, P. G. M. Wuts, *Protective groups in organic synthesis*, Wiley, New York, **1999**.
[91] M. J. Moonen, I. M. Rietjens, W. J. van Berkel, *J. Ind. Microbiol. Biotechnol.* **2001**, *26,* 35 – 42.
[92] M. Held, A. Schmid, H.-P. Kohler, W. Suske, B. Witholt, M. G. Wubbolts, *Biotechnol. Bioeng.* **1999**, *62,* 641 – 648.
[93] L. E. Husken, M. C. Dalm, J. Tramper, J. Wery, J. A. de Bont, R. Beeftink, *J. Biotechnol.* **2001**, *88,* 11 – 19.
[94] W. Wouters, M. T. Tulp, P. Bevan, *Eur. J. Pharmacol.* **1988**, *149,* 213 – 223.
[95] I. Ekaeva, L. Barre, M.-C. Lasne, F. Gourand, *Appl. Radiat. Isot.* **1995**, *46,* 777 – 782.
[96] A. J. M. da Silva, P. A. Melo, N. M. V. Silva, F. V. Brito, C. D. Buarque, D. V. de Souza, V. P. Rodrigues, E. S. C. Pocas, F. Noel, E. X. Alburquerque, P. R. R. Costa, *Bioorg. Med. Chem. Lett.* **2001**, *11,* 283 – 286.
[97] D. P. Ivanov, V. I. Sobolev, L. V. Pirutko, G. I. Panov, *Adv. Synth. Catal.* **2002**, *344,* 986 – 995.
[98] N. Ran, D. R. Knop, K. M. Draths, J. W. Frost, *J. Am. Chem. Soc.* **2001**, *123,* 10927 – 10934.
[99] M. C. Carreno, *Chem. Rev.* **1995**, *95,* 1717 – 1760.
[100] B. R. Bochner, B. N. Ames, *J. Biol. Chem.* **1982**, *257,* 9759 – 9769.
[101] C. L. Elmore, T. D. Porter, *J. Biol. Chem.* **2002**, *277,* 48960 – 48964.
[102] M. H. M. Eppink, K. M. Overkamp, H. A. Schreuder, W. J. H. Van Berkel, *J. Mol. Biol.* **1999**, *292,* 87 – 96.
[103] P. Morandi, B. Valzasina, C. Colombo, B. Curti, M. A. Vanoni, *Biochemistry* **2000**, *39,* 727 – 735.

[104] N. S. Scrutton, A. Berry, R. N. Perham, *Nature* **1990**, *343,* 38 – 43.
[105] N. M. Kamerbeek, D. B. Janssen, M. W. Fraaije, in *Flavins and Flavoproteins 2002,* (Eds.: S. K. Chapman, R. Perham, N. S. Scrutton), Agency for Scientific Publication, Berlin, **2002**.
[106] M. W. Fraaije, N. M. Kamerbeek, W. J. H. van Berkel, D. B. Janssen, *FEBS Lett.* **2002**, *518,* 43 – 47.
[107] T. A. Vannelli, A. Dykman, P. R. Ortiz de Montellano, *J. Biol. Chem.* **2002**, *277,* 12824 – 12829.
[108] S. Hartmans, J. A. M. de Bont, *FEMS Microbiol. Lett.* **1986**, *36,* 155 – 158.
[109] C. Asselineau, J. Asselineau, G. Laneelle, M.-A. Laneelle, *Progr. Lipid Res.* **2002**, *41,* 501 – 523.
[110] K. Yabe, N. Chihaya, S. Hamamatsu, E. Sakuno, T. Hamasaki, H. Nakajima, J. W. Bennett, *Appl. Environ. Microbiol.* **2003**, *69,* 66 – 73.
[111] Z. Zhang, A. A. Schaffer, W. Miller, T. L. Madden, D. J. Lipman, E. V. Koonin, S. F. Altschul, *Nucleic Acids Res.* **1998**, *26,* 3986 – 3990.
[112] G. P. Chen, L. L. Poulsen, D. M. Ziegler, *Drug. Metab. Dispos.* **1995**, *23,* 1390 – 1393.
[113] D. M. Ziegler, *Drug Metabol. Rev.* **2002**, *34,* 503 – 511.
[114] J. W. Eckstein, J. W. Hastings, S. Ghisla, *Biochemistry* **1993**, *32,* 404 – 411.
[115] R. Villa, A. Willetts, *J. Mol. Cat. B: Enzym.* **1997**, *2,* 193 – 197.
[116] L. Prado, E. Fernandez, U. Weissbach, G. Blanco, L. M. Quiros, A. F. Brana, C. Mendez, J. Rohr, J. A. Salas, *Chem. Biol.* **1999**, *6,* 19 – 30.
[117] A. Li, J. Piel, *Chem. Biol.* **2002**, *9,* 1017 – 1026.
[118] M. H. M. Eppink, H. A. Schreuder, W. J. H. van Berkel, *Protein Sci.* **1997**, *6,* 2454 – 2458.
[119] C. S. Orser, C. C. Lange, L. Xun, T. C. Zahrt, B. J. Schneider, *J. Bacteriol.* **1993**, *175,* 411 – 416.
[120] M. H. Eppink, H. A. Schreuder, W. J. van Berkel, *J. Biol. Chem.* **1998**, *273,* 21031 – 21039.
[121] J. Wang, M. Ortiz-Maldonado, B. Entsch, V. Massey, D. Ballou, D. L. Gatti, *Proc. Natl. Acad. Sci. USA* **2002**, *99,* 608 – 613.
[122] M. K. Trower, R. M. Buckland, M. Griffin, *Eur. J. Biochem.* **1989**, *181,* 199 – 206.
[123] G. J. Lye, P. Ayazi-Shamlou, F. Baganz, P. A. Dalby, J. M. Woodley, *Trends Biotechnol.* **2003**, *21,* 29 – 37.
[124] A. Meyer, A. Schmid, M. Held, A. H. Westphal, M. Rothlisberger, H. P. Kohler, W. J. H. van Berkel, B. Witholt, *J. Biol. Chem.* **2002**, *277,* 5575 – 5582.
[125] A. Meyer, M. Wursten, A. Schmid, H. P. Kohler, B. Witholt, *J. Biol. Chem.* **2002**, *277,* 3461 – 34167.
[126] A. B. Watts, J. Beecher, C. S. Whitcher, J. A. Littlechild, *Biocatalysis and Biotrans.* **2002**, *20,* 209 – 215.
[127] P. W. Trudgill, *Methods Enzymol.* **1990**, *188,* 77 – 81.
[128] G. Carrea, B. Redigolo, S. Riva, S. Colonna, N. Gaggero, E. Battistel, D. Bianchi, *Tetrahedron Asymmetry* **1992**, *3,* 1063 – 1068.

