Baeyer-Villiger monooxygenases, an emerging family of flavin-dependent biocatalysts

Nanne M. Kamerbeek, Dick B. Janssen, Willem J. H. van Berkel, Marco W. Fraaije

ABSTRACT

Baeyer-Villiger monooxygenases (BVMO) 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 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 have been sequenced and overexpressed. In this paper the biocatalytic properties of recently cloned BVMOs have been sequenced and overexpressed. Furthermore, the potential of obtaining novel BVMOs from sequenced genomes will be discussed.

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 (Baeyer and Villiger, 1899). In this reaction, the ketone is attacked by a nucleophilic peroxy acid to form the so-called tetrahedral ‘Criegee intermediate’ (Scheme 1) (Criegee, 1948). 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 (Renz and Meunier, 1999). 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 has several disadvantages that do not match with the principles of green chemistry (Anastas et al., 2000). 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.
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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 (Strukul, 1998) and organocatalytic compounds (Fukuda et al., 2001; Mazzini et al., 1996; Murahashi et al., 2002) 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 CO₂ as a solvent, establishing Baeyer-Villiger oxidation of various ketones using oxygen as primary oxidant and benzaldehyde or pivalaldehyde as co-reductant (Bolm et al., 2002). 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 (Turfitt, 1948). Since then, Baeyer-Villiger oxidation steps have been found in biosynthetic pathways in many different organisms e.g.: aflatoxin synthesis in fungi (Townsend et al., 1982), synthesis of iridoids and steroids in plants (Damtoft et al., 1995; Winter et al., 1999), and toxin synthesis in shellfish (Wright et al., 1996). 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 (Britton and Markovetz, 1977; Forney and Markovetz, 1969), alicyclic hydrocarbons (Donoghue and Trudgill, 1975; Griffin and Trudgill, 1976; Schumacher and Fakoussa, 1999), aromatic compounds (Casellas et al., 1997; Cripps, 1975; Cripps et al., 1978; Darby et al., 1987; Havel and Reineke, 1993; Higson and Focht, 1990; Hopper et al., 1985; Jones et al., 1994), and terpenes (Ougham et al., 1983; van der Werf and Boot, 2000).

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 composed of α₂β trimers (Willetts, 1997). 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 (Colonna et al., 1996; Flitsch and Grogan, 2002; Mihovilovic et al., 2002b; Roberts and Wan, 1998; Walsh and Chen, 1988; Willetts, 1997)). 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) (Iwaki et al., 2002), cyclododecanone monooxygenase (CDMO; EC 1.14.13.x) (Kostitchka et al., 2001), steroid monooxygenase (SMO; EC 1.14.13.54) (Mori et al., 1999), and 4-hydroxyacetophenone monooxygenase (HAPMO; EC 1.14.13.x) (Kamerbeek et al., 2001). 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 BVMOs

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 (Y. C. Chen et al., 1988). The CHMO sequence deposited later by Iwaki et al. (Iwaki et al., 1999b) differed from that originally reported by Chen et al. at several nucleotide positions (Cheesman et al., 2001). Recent mass spectrometry experiments proved that the sequence deposited by Iwaki et al. is the correct CHMO sequence (Kneller et al., 2001). The CHMO gene encodes a 60.9 kDa protein appearing as monomer upon purification (Table 1). Recently, six other cyclohexanone monooxygenase genes were identified (Brzostowicz et al., 2000; Brzostowicz et al., 2003; Cheng et al., 2000). All these newly identified CHMOs show significant protein sequence identity with CHMO from Acinetobacter NCIMB 9871. While activity with cyclohexanone 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 (Ryerson et al., 1982; Sheng et al., 2001). 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 (Walsh and Chen, 1988). The electrophilic hydro peroxyflavin catalyzes asymmetric sulfoxidation of various thioethers (G. Chen et al., 1999), oxidation of amines (Colonna et al., 2003; Ottolina et al., 1999), and oxidation of selenides (Walsh and Chen, 1988) (Scheme 2). The nucleophilic peroxyflavin catalyzes not only Baeyer-Villiger reactions but is also responsible for
Table 1. Biochemical properties of the characterized Type I BVMOs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>year of cloning</th>
<th>Catalyzed reaction</th>
<th>Spec. Act.</th>
<th>$K_{M,S}$</th>
<th>$K_{M,NADPH}$</th>
<th>Subunit Mw</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHMO</td>
<td>1988</td>
<td>cyclohexanone → caprolactone</td>
<td>30</td>
<td>4</td>
<td>20</td>
<td>60.9</td>
<td>(Ryerson et al., 1982)</td>
</tr>
<tr>
<td>CPMO</td>
<td>2002</td>
<td>cyclopentanone → valerolactone</td>
<td>4.3</td>
<td>&lt; 1</td>
<td>&lt; 3</td>
<td>62.1</td>
<td>(Iwaki et al., 2002; Trudgill, 1990)</td>
</tr>
<tr>
<td>CDMO</td>
<td>2001</td>
<td>cyclododecanone → lauryl lactone</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>67.5</td>
<td>(Kostichka et al., 2001)</td>
</tr>
<tr>
<td>SMO</td>
<td>1999</td>
<td>progesterone → 17-O-acetyl-testosterone</td>
<td>14</td>
<td>55</td>
<td>0.44</td>
<td>60.1</td>
<td>(Morii et al., 1999)</td>
</tr>
<tr>
<td>HAPMO</td>
<td>2001</td>
<td>4-hydroxyacetophenone → 4-hydroxyphenyl acetate</td>
<td>10.5</td>
<td>9.2</td>
<td>64</td>
<td>71.9</td>
<td>(Kamerbeek et al., 2001; Kamerbeek et al. 2003a)</td>
</tr>
</tbody>
</table>

[a] 1 Unit is defined as the amount of enzyme that oxidizes 1 µmol NADPH / min.
the observed boron oxidation reactions (Branchaud and Walsh, 1985). The recently reported CHMO-mediated asymmetric epoxidation reactions were also suggested to proceed via the nucleophilic peroxyflavin species (Colonna et al., 2002), although it has been proposed that the electrophilic hydroperoxyflavin could catalyze epoxidation reactions (Branchaud and Walsh, 1985).

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 (Stewart, 1998b), various sulfoxides (G. Chen et al., 1999; Colonna et al., 1996), cyclic sulfates (Colonna et al., 1998) and thiosulfinates (Colonna et al., 2001). CHMO displays a remarkable 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 (Berezina et al., 2002) complying to the need of transformations with (theoretically) 100% yield and 100% ee (Strauss et al., 1999). 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 (Mihovilovic et al., 2002b).

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 (Hummel, 1999). 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 (Tishkov et al., 1993) and Saccharomyces cerevisiae (Serov et al., 2002) have been engineered from NAD\(^+\) towards NADP\(^+\) preferring enzymes. The Pseudomonas enzyme has been used in combination with CHMO-catalyzed conversions (Rissom et al., 1997; Schwarz-Linek et al., 2001). Zambianchi et al. tested different NADPH regeneration systems and found optimal results with 2-propanol/alcohol dehydrogenase from Thermoanaerobium brockii (Zambianchi et al., 2002). An alternative approach for coenzyme recycling is represented by electrochemical regeneration of coenzymes (Somers et al., 1997). For example, the pentamethylcyclopentadienyl rhodium bipyridine complex \([\text{Cp}^*\text{Rh(bpy)(H}_2\text{O})]^2^+\) is reduced by electrons from the cathode and takes up a proton yielding \([\text{Cp}^*\text{Rh(bpy)}\text{H}]^+\) which acts as a hydride transfer reagent on NAD(P)\(^+\). This system has successfully been used for synthetic application of a NADH-dependent monooxygenase (Hollmann et al., 2001; Hollmann et al., 2002).

Although isolated enzymes have certain advantages, whole-cell conversions with oxygenases are attractive for several reasons (Duetz et al., 2001). One major advantage is the efficient intracellular coenzyme regeneration. Furthermore, in 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 (G. Chen et al., 1999; S.D. Doig et al., 2001) and expression in yeast has also been established (Cheesman et al., 2001; Stewart et al., 1996). Whole-cell conversions using recombinant CHMO-containing E. coli cells has been optimized and applied by Walton and Stewart (Walton and Stewart, 2002), who efficiently converted cyclohexanone into \(\epsilon\)-caprolactone. Furthermore, Doig et al. (S. D. Doig et al., 2002), 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-oxabicyclo-oct-6-en-3-one lactones are valuable intermediates for synthesis of prostaglandins (Alphand et al., 1989; Newton and Roberts, 1980).

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.
2.2 Cyclopentanone monooxygenase (CPMO)

CPMO was purified in 1976 by Griffin et al. from Comamonas (previously Pseudomonas) sp. NCIMB 9872 growing on cyclopentanone (Griffin and Trudgill, 1976). 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 (Iwaki et al., 2002). 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 (Bes et al., 1996; Kelly et al., 1996; Sandey and Willetts, 1989). Initial tests showed that the enzyme prefers C₄ to C₈ ketones and norbornanone (2) as substrates (Griffin and Trudgill, 1976). 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 = CH₃) is converted by CHMO to the (S)-lactone whereas CPMO produces the (R)-lactone (Iwaki et al., 2002). Another example is the conversion of racemic 2-substituted cyclopentanones (4). While the CPMO-catalyzed conversions of these cyclopentanones are non-enantioselective (Iwaki et al., 2002), CHMO shows high enantioselectivity with these compounds, enabling kinetic resolution (Kayser et al., 1998). 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 (Bes et al., 1996). These conversions are of interest because many functionalized chiral δ-valerolactones are biologically active compounds and valuable intermediates in natural product synthesis (Coutrot et al., 1999).

CPMO is also able to catalyze oxidation of the prochiral fused system (5) forming the opposite enantiomer to that obtained with CHMO (Mihovilovic et al., 2002; Mihovilovic et al., 2002b). 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 and 5-methoxy-indan-1-one (7,8) could only be converted by CPMO (Iwaki et al., 2002). The same result was recently found by Furstoss and co-workers who tested various substituted 1-indanones with whole cells expressing CHMO, CPMO or HAPMO. Only CPMO-expressing cells converted some of the tested indanones (Gutierrez et al., 2003). Also 4-methyl-4-n-propylcyclohexanone (9) is only a substrate for CPMO (Iwaki et al., 2002). 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 (Cheng et al., 2002). In 1999, the first cyclododecanone monooxygenase (CDMO) from Rhodococcus ruber CD4 was purified and characterized
(Schumacher and Fakoussa, 1999). 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* (Kostichka et al., 2001). 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\textsubscript{11}-C\textsubscript{15} cyclic ketones. Insignificant conversion was observed with C\textsubscript{6} and C\textsubscript{10} cyclic ketones and the enzyme was inactive towards C\textsubscript{7} and C\textsubscript{8} cyclic ketones (Kostichka et al., 2001). 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 (Thomas et al., 2002).

### 2.4 Steroid monooxygenase (SMO)

As mentioned in the introduction, the first discovered BVMO activities involved microbial conversion of steroids (Turfitt, 1948). Two steroid monooxygenases performing Baeyer-Villiger reactions have been purified and characterized (Itagaki, 1986; Miyamoto et al., 1995). 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), the fungal enzyme also catalyzes oxidative lactonization of androstenedione to testololactone (Itagaki, 1986). A recombinant expression system for *R. rhodochrous* SMO has been constructed in *E. coli* (Morii et al., 1999). 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 (Biellmann, 2002).

### 2.5 4-Hydroxyacetophenone monooxygenase (HAPMO)

Microbial Baeyer-Villiger oxidation of aromatic compounds was first reported in the mid 70’s (Cripps, 1975). Several of these aromatic degradation pathways involving Baeyer-Villiger oxidation have been elucidated which include the catabolic routes for acetophenones (Cripps, 1975; Cripps et al., 1978; Havel and Reineke, 1993; Higson and Focht, 1990), 1-phenyl ethanol (Cripps et al., 1978), 4-ethylphenol (Jones et al., 1994), and fluorine (Casellas et al., 1997). The first purification of a BVMO active on aromatic compounds was only described in 1999 (Moonen et al., 1999). This enzyme, 4-hydroxyacetophenone monooxygenase (HAPMO), was isolated from *Pseudomonas fluorescens* ACB growing on 4-hydroxyacetophenone (Table 1) (Higson and Focht, 1990).
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 (Tanner and Hopper, 2000). HAPMO from *P. fluorescens* ACB is a homodimer of 144 kDa with each subunit containing a tightly non-covalently bound FAD. In *P. fluorescens* ACB, the enzyme oxidizes 4-hydroxyacetophenone towards 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* (Kamerbeek et al., 2001). 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 (Kamerbeek et al., 2001).

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 (Kamerbeek et al., 2001). 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 (Kamerbeek et al., 2003). 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 hydroxyl group(s) to prevent oxidation reactions. For this, ethers are the most widely used protective groups, while esterification is an important alternative (Greene and Wuts, 1999). 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) (Moonen et al., 2001).

![Scheme 3. Biocatalytic production of protected catechols using HAPMO.](image)

Substituted catechols are valuable precursors for the synthesis of pharmaceutical compounds (Held et al., 1999; Husken et al., 2001; Wouters et al., 1988). 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. Recent $^{19}$F NMR studies also 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 (Moonen et al., 2001).

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) (Ekaeva et al., 1995). Substituted phenols obtained by chemical Baeyer-Villiger oxidation of aromatic aldehydes can also be used as building blocks for the synthesis of different coumestans, which are biologically active flavonoids (da Silva et al., 2001). Furthermore, hydrolysis of 4-hydroxyphenyl acetate affords hydroquinone, which is like other dihydroxybenzenes, an important intermediate of organic synthesis (Ivanov et al., 2002). 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 (Ran et al., 2001).

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 (Carreno, 1995). HAPMO performs highly enantioselective oxidation of methyl phenyl sulfide (10) and methyl $p$-tolyl sulfide (Kamerbeek et al., 2003). Interestingly, compared to CHMO and CPMO, HAPMO shows a better performance in methyl $p$-tolyl sulfide oxidation (Table 2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>HAPMO (Kamerbeek et al., 2003a)</th>
<th>CHMO (Carrea et al., 1992)</th>
<th>CPMO (Kelly et al., 1996)$^{[a]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>methyl phenyl sulfide</td>
<td>$&gt; 99 %$ (S)</td>
<td>99 % (R)</td>
<td>100 % (S)</td>
</tr>
<tr>
<td>methyl $p$-tolyl sulfide</td>
<td>$&gt; 99 %$ (S)</td>
<td>37 % (S)</td>
<td>84 % (S)</td>
</tr>
</tbody>
</table>

$^{[a]}$ Experiments performed with whole cells.

In contrast to CHMO (Alphand et al., 1989), 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 $(1R, 5S)$ bicyclohept-2-en-6-one above the $(1S, 5R)$ enantiomer exhibiting an $E$-value of 20. Therefore, HAPMO can be used for a kinetic resolution to obtain the $(1S, 5R)$ enantiomer (Kamerbeek et al., 2003a).
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 (Bochner and Ames, 1982; Elmore and Porter, 2002). For several flavoproteins a switch in coenzyme specificity has been achieved by enzyme engineering (M. H. M. Eppink et al., 1999; Morandi et al., 2000; Scrutton et al., 1990), but no Baeyer-Villiger monoxygenase 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 (Kamerbeek et al., 2004). 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 BVMOs

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 (Fraaije et al., 2002). This revealed that Type I BVMOs are part of a superfamily of sequence-related flavin dependent monoxygenases. 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 monoxygenase subfamilies can be recognized within this novel superfamily: (1) the so-called FMO family mainly consisting of heteroatom-oxidizing monoxygenases from eukaryotic origin, (2) a family of bacterial amine-hydroxylating monoxygenases (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 (Fraaije et al., 2002). 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 (Fraaije et al., 2002).

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).

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

<table>
<thead>
<tr>
<th>organism</th>
<th>total number of genes</th>
<th>number of Type I BVMO genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>4289</td>
<td>0</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>3924</td>
<td>6</td>
</tr>
<tr>
<td>M. leprae TN</td>
<td>1605</td>
<td>1</td>
</tr>
<tr>
<td>S. coelicolor A3(2)</td>
<td>7512</td>
<td>2</td>
</tr>
<tr>
<td>P. aeruginosa PAO1</td>
<td>5565</td>
<td>3</td>
</tr>
<tr>
<td>S. cerevisae</td>
<td>6449</td>
<td>0</td>
</tr>
</tbody>
</table>

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 (Vannelli et al., 2002). 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. in prep). This is in line with the observation that Mycobacteria catalyze a variety of Baeyer-Villiger reactions (Asselineau et al., 2002; Hartmans and de Bont, 1986). 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 (Yabe et al., 2003). 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 where 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 (Zhang et al., 1998). 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/) performed 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 have 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 (G. P. Chen et al., 1995). This enzyme was found to belong to the above-mentioned FMO family and therefore is related to Type I BVMOs (Fraaije et al., 2002). 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 (Ziegler, 2002). 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 (Fraaije et al., 2002) 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 (Damtoft et al., 1995; Winter et al., 1999), 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 peroxymethacetal upon the attack of the peroxyflavin on the substrate (Eckstein et al., 1993). Instead of the Baeyer-Villiger rearrangement, a chemically initiated electron luminescence (CIEEL) mechanism has been proposed (Eckstein et al., 1993). Nonetheless, the enzyme is able to convert bicycloheptenone via a Baeyer-Villiger type of reaction (Villa and Willetts, 1997). 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 (Li and Piel, 2002; Prado et al., 1999). Recently, a gene from Streptomyces has been identified to catalyze the oxidative cleavage of the premithramycin B at the expense of NADPH and
Chapter 7

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 (M. H. M. Eppink et al., 1997; Orser et al., 1993). 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 (M. H. Eppink et al., 1998; Wang et al., 2002). 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 (Trower et al., 1989). 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 (Lye et al., 2003).

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 have already been reported for a flavoprotein monooxygenase for which structural data are lacking (A. Meyer et al., 2002b; A. Meyer et al., 2002). Therefore, the availability of sequence related BVMO genes will allow fine-tuning of catalytic properties by exploiting random mutagenesis and gene shuffling 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 (Watts et al., 2002). Taken together, the above-mentioned recent developments will enable strategies to design tailor-made Baeyer-Villiger biocatalysts in the near future.