Redesign of Baeyer–Villiger Monooxygenases for Synthetic Applications
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Chapter 2

Baeyer–Villiger monooxygenases:
Recent advances and future challenges

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Abstract

For many enzyme classes, a wealth of information on structure and mechanism has been generated in the last few decades. While the first Baeyer–Villiger mono-oxygenases (BVMOs) were isolated more than 30 years ago, detailed data on these enzymes was lacking until recently. Over the last years, several major scientific breakthroughs, including the elucidation of BVMO crystal structures and the identification of numerous novel BVMOs, have boosted the research on BVMOs. This has led to intensified biocatalytic exploration of novel BVMOs and structure-inspired enzyme redesign. This chapter provides an overview on the lately gained knowledge on BVMOs and sketches the outlook for future industrial applications of these unique oxidative biocatalysts.
Baeyer–Villiger oxidation: Chemical and biochemical methods

In 1899, Adolf Baeyer and Victor Villiger described an oxidation reaction in which carbonylic compounds are converted into the corresponding esters or lactones. Hence, this atypical reaction has been named Baeyer–Villiger (B–V) oxidation. Nowadays, B–V oxidations are frequently employed in synthetic organic chemistry (Renz and Meunier, 1999; ten Brink et al., 2004). However, due to the lack of selectivity of chemical catalysts and the harsh conditions needed for catalysis, the enzymatic counterparts are attractive to use as biocatalysts. The first Baeyer–Villiger monooxygenase (BVMO) for which the synthetic potential was demonstrated is cyclohexanone monooxygenase (CHMO\textsubscript{Acinetobacter}) from \textit{Acinetobacter} sp. strain NCIB 9781 (Donoghue et al., 1976). While CHMO\textsubscript{Acinetobacter} is produced by the bacterium for catalysing the first step in cyclohexanone degradation, it has been shown to accept hundreds of different carbonylic compounds, displaying an exceptionally broad substrate acceptance profile in combination with very high regio- and/or enantioselectivity (Mihovilovic et al., 2002; Stewart, 1998). Further studies proved that various types of oxidations could be catalysed by CHMO\textsubscript{Acinetobacter} and other BVMOs (Colonna et al., 1996; Ottolina et al., 1999; Secundo et al., 1993). These features render BVMOs as attractive oxidative biocatalysts. Efforts to identify and catalytically exploit these enzymes have led in the last 15 years to the availability of new recombinant BVMOs for biocatalysis (Figure 1). This review provides a summary of recent developments in discovery, structural and redesign studies as well as applications of BVMOs in organic synthesis. For more details on their biocatalytic scope, we refer to other reviews (de Gonzalo et al., 2010; Kayser, 2009; Leisch et al., 2011).

Biodiversity of BVMOs

Along with the identification of several genes encoding BVMOs at the beginning of this century (Iwaki et al., 2002; Kamerbeek et al., 2001; Kostichka et al., 2001; Morii et al., 1999; van Beilen et al., 2003), it was realised that they form a sequence related enzyme family (Type I BVMOs) of FAD-containing and NADPH-dependent enzymes of which members can be identified with a specific protein-sequence motif (Fraaije et al., 2002). Although the molecular basis for the conservation of these specific residues remained obscure until recently (\textit{vide infra}), the sequence motif has been very useful for mining genomes for novel BVMOs. It has revealed a striking distribution of these enzymes among organisms. While bacteria and fungi are relatively rich in BVMOs, containing on average one BVMO/genome, only few Type I BVMOs are present in plant and animal genomes. Among bacteria, these enzymes are prevalent among actinomycetes, rendering these bacteria an interesting source for novel BVMOs whilst
also fungal genomes are relatively rich in BVMOs and mostly unexplored. Recent studies on the biotransformation of natural products confirm the vital roles that BVMOs play in microbial metabolic pathways. BVMOs catalyse key steps in degradation of, among others, acetone (Kotani et al., 2007), bulky cyclic aliphatic ketones (Iwaki et al., 2006; Kostichka et al., 2001), and linear ketones (Kirschner et al., 2007; Rehdorf et al., 2007; Völker et al., 2008) (Figure 2A). In addition, highly complex biomolecules are tailored by BVMOs as exemplified by the conversion of steroids (Beneventi et al., 2009; Kolek et al., 2008; Świzdor et al., 2011) and biosynthesis of sesquiterpenoids (Jiang et al., 2009; Seo et al., 2011) and aflatoxins (Wen et al., 2005) (Figure 2A). Except for being highly suitable for biocatalytic applications, BVMOs can be of medical relevance as well. The \( \text{etaA} \) gene of \( \text{Mycobacterium tuberculosis} \) has been shown to be responsible for activating thiocarbamide prodrugs (Dover et al., 2007), and mutations in this gene make \( \text{M. tuberculosis} \) drug-resistant. Heterologous expression of EtaA enabled us to establish that it represents a \( \text{bona fide} \) BVMO acting on a number of ketones (Fraaije et al., 2004). It has been demonstrated that by designing drugs for upregulation of the \( \text{etaA} \) gene, efficacy of antitubercular prodrugs can be enhanced (Willand et al., 2009). This indicates that BVMOs can be highly interesting as (pro)drug target as they are prevalent in proteomes of pathogenic microbes while no BVMO gene is present in the human genome.

A BVMO related to EtaA was isolated from \( \text{Acinetobacter radioresistens} \) strain S13 (Minerdi et al., 2012). The expression of this enzyme is induced during the growth of the organism in the presence of long-chain alkanes (C24, C36). Therefore, this enzyme is implicated to play a role in subterminal oxidation of alkanes. The activity of this BVMO on 4-phenyl-2-butanone and ethionamide was
confirmed. Further characterisation of this new enzyme would be worthwhile in order to investigate its substrate scope.

Recently, 4-sulfoacetophenone monooxygenase (SAPMO) was cloned from Comamonas testosteroni KF-1 (Weiss et al., 2012). SAPMO is involved in biodegradation of surfactant linear alkylbenzenesulfonate and shares above 55% sequence homology with phenylacetone monooxygenase (PAMO) and steroid monooxygenase (STMO). SAPMO was found to be strictly NADPH-dependent, and it accepted as substrates several aromatic ketones and benzaldehyde while neither progesterone, acetone, nor cyclohexanone was converted. However, the enzyme is poorly stable as half of the activity was lost after 1 h at 30 °C. Notably, Comamonas testosteroni KF-1 contains three other putative BVMO genes.

Interestingly, a domain of a hybrid polyketide synthase/nonribosomal peptide synthetase was identified to be a Type I BVMO (Tang et al., 2013). This domain catalyses the B–V oxidation of an acyl carrier protein-tethered thioester to a thio-carbonate in the synthesis of antitumour compound FR901464 and represents the first example of a BVMO domain acting within a polyketide synthase/nonribosomal peptide synthetase system.

In the last few years, three BVMOs involved in the degradation of camphor in Pseudomonas putida NCIMB 10007 have been cloned and characterised. The degradation of camphor starts with hydroxylation by a P450 monooxygenase, followed by oxidation by an alcohol dehydrogenase to diketocamphane, and subsequent oxidation by Type II BVMOs. Two enzymes are needed for the oxidation of two isomers of camphor: 2,5-diketocamphane 1,2-monooxygenase (2,5-DKCMO) is involved in the degradation of (+)-camphor, while (−)-camphor requires 3,6-diketocamphane 1,6-monooxygenase (3,6-DKCMO). B–V oxidation of both diketocamphanes leads to the same unstable lactone product which decays to 2-oxo-Δ^3^-4,5,5-trimethylcyclopentenylacetic acid. The latter product is modified by CoA and becomes a substrate of another BVMO, 2-oxo-Δ^1^-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase (OTEMO). Kadow et al. reported on cloning and expression of the oxygenating subunit of 2,5-DKCMO (Kadow et al., 2011). In a subsequent study, OTEMO and the oxygenating subunit of 3,6-DKCMO were cloned, expressed, and purified with good yields (Kadow et al., 2012). These three enzymes displayed the highest activity with bicyclic ketones such as (+)- and (−)-camphor, bicyclo[3.2.0]-hept-2-en-6-one, and norcamphor. 3,6-DKCMO and OTEMO showed a broader substrate range than 2,5-DKCMO, being able to convert some cycloaliphatic and aromatic ketones as well. A more thorough characterisation of OTEMO was performed by the Lau group who also solved the structure of this enzyme (Leisch et al., 2012). 2-oxo-Δ^1^-4,5,5-trimethylcyclopentenylacetyl-CoA was the best substrate of OTEMO in terms of $K_M$, while the acid form was a very poor
substrate. Several substituted cyclopentanones and 2-oxopentyl or 2-oxohexyl esters were converted. *Escherichia coli* cells expressing OTEMO were used in the kinetic resolution of 2-methylcyclopentanone yielding a high $E$ value ($> 200$) while only a low $E$ value was achieved when CHMO from *Rhodococcus* sp. strain HI-31 (CHMO$_{RhodoHI-31}$) was used as a reference enzyme. In the kinetic resolution of 4-substituted cyclohexanones catalysed by OTEMO, high ee values and often complementary enantiopreference, as compared to CHMO$_{RhodoHI-31}$ and cyclopentanone monoxygenase (CPMO), were achieved. While OTEMO presented some interesting biocatalytic properties, and in contrast to 1,2-DKCMO and 3,6-DKCMO, it does not require a reductase unit, the stability of OTEMO was found to be rather limited, as its half-life at 40 °C was found to be only 3.8 min.

Despite several reports on B–V reaction catalysed by different fungal species, no eukaryotic genes were cloned or proteins characterised until recently. In 2012, the Bornscheuer group succeeded in cloning the first BVMO-encoding gene of eukaryotic origin from the ascomycete *Cylidrocarpon radicicola* ATCC 11011, which was known to convert progesterone and bicyclic ketones (Leipold et al., 2012). This BVMO, named cycloalkane monooxygenase (CAMO), shares homology with known CHMOs and is active on cyclic aliphatic and bicyclic ketones. Low activity on open chain ketones was observed, while steroids were not converted. The last observation suggests that at least one more BVMO is produced by this organism.

**Atypical BVMOs**

While most of the reported BVMOs are sequence related, some BVMOs do not resemble these so-called Type I BVMOs. One of the atypical BVMOs is MtmOIV which represents a new type of FAD- and NADPH-dependent BVMOs that acts as a key enzyme in the biosynthetic pathway of mithramycin, a polyketide anticancer agent. The elucidation of its crystal structure and structure-based mutagenesis studies have identified several residues that are involved in substrate binding, although the crystal structures did not reveal the exact binding of either NADP(H) or mithramycin (Beam et al., 2009). Unfortunately, MtmOIV displays narrow substrate specificity and hence appears less relevant for biocatalysis.

Another atypical BVMO, GilOII from a *Streptomyces* species, was shown to catalyse a series of reactions in the biosynthesis of gilvocarcin (an antitumour compound): hydroxylation of dehydrorabelomycin, B–V oxidation leading to hydroxyoxepinone intermediate, and finally a C–C bond cleavage (Tibrewal et al., 2012). GilOII requires FAD, NADPH, and a flavin reductase, and it shares no homology with Type I or II BVMOs. A related protein JadG performs the same sequence of reactions in the biosynthesis of jadomycin.
Several FMOs classified as Type II FMOs were recently reported to catalyse B–V oxidations. FMO from *Stenotrophomonas maltophilia* (Jensen et al., 2012) converted bicyclo[3.2.0]-hept-2-en-6-one while three FMOs from *Rhodococcus jostii* RHA1 catalysed B–V oxidations of bicyclo[3.2.0]-hept-2-en-6-one and phenylacetone (Riebel et al., 2013). A common feature that sets Type II FMOs apart from Type I FMOs and Type I BVMOs is their relaxed coenzyme specificity: these FMOs are active with both NADPH and NADH. In addition, human FMO5 was found to catalyse a B–V oxidation of a ketone drug metabolite (Lai et al., 2011).

*Tapping bacterial genomes for BVMOs*

In order to obtain a robust BVMO, we used the BVMO-specific sequence motif to identify and clone a gene from the mesotherophilic actinomycete *Thermobifida fusca*. The respective enzyme, PAMO, could be easily expressed in *E. coli* and was shown to be primarily active with aromatic compounds (Fraaije et al., 2005). Several studies have established that the enzyme can be effectively used for regio- and enantioselective conversions of aromatic compounds (Ríoz-Martínez et al., 2009; Rodríguez et al., 2007; Rodríguez et al., 2009). Another attractive feature of PAMO is its stability towards higher temperatures and a range of organic solvents. In fact, the use of solvents can also improve the enantioselective behaviour (Rodríguez et al., 2008).

The direct harvesting of novel BVMOs by exploring sequenced genomes has been intensified by the Grogan group. By using effective ligation-independent cloning, they have reported on cloning of 29 Type I BVMO genes from the actinomycetes *Mycobacterium tuberculosis* (Bonsor et al., 2006) and *R. jostii* (Szolkowy et al., 2009). This resulted in soluble expression of most of the corresponding enzymes, and for many of them BVMO activity could be confirmed. The set of rhodococcal BVMOs was thoroughly characterised by Riebel et al. (2012). This work provided more accurate annotation of BVMOs and expression conditions for all 22 BVMOs. The activity of these enzymes was tested on 39 chemically diverse substrates including linear aliphatic ketones, cyclic aliphatic ketones, aromatic ketones, aromatic amines, and aromatic sulfides. For 14 enzymes, activity on at least one substrate was demonstrated while 5 enzymes proved to be “potent BVMOs” as they are active on 10 or more compounds. Furthermore, a new BVMO-typifying fingerprint motif was identified. This motif \([A/G]\)GxWxxxx\([F/Y]\)P\([G/M]\)xxxD is found in all known Type I BVMOs between the N-terminal GxGxxG motif and the previously described BVMO motif. It is specific for BVMOs and allows discrimination between BVMOs and FMOs. Clearly, the approach of sifting through sequenced genomes for novel BVMOs is productive and can be improved thanks to the new sequence motif.
Figure 2. A. Some newly identified BVMO substrates: acetone, cyclopentadecanone, androstenedione (steroid), 1-deoxy-11-oxopentalenic acid (sesquiterpenoid precursor), and hydroxyversicolorone (aflatoxin precursor). The site of oxygen insertion is indicated with an arrow. B. A schematic view of the BVMO mechanism as elucidated for CHMO<sub>Acinetobacter</sub> and PAMO. The peroxyflavin intermediate is shown in red, reduced flavin in green, and oxidised flavin in blue.

Figure 3. Crystal structure of PAMO with the FAD cofactor and R337 highlighted in sticks (1W4X). The FAD-binding domain is in green, the NADPH domain is in blue, R337 is shown in two conformations as observed in the crystal structure. The insets show details of the active sites of PAMO, CHMO<sub>Rhodohalobacter</sub>, in the closed form (3GWD), and CHMO<sub>Rhodobacter</sub> in the open form (3GWF). NADP<sup>+</sup> is shown in magenta. Some key residues and the bulge are indicated. The figure was prepared with the PyMol software.
Structural basis for biocatalytic B–V oxidations

The sudden increase in discovery and characterisation of BVMOs eventually led to the elucidation of the first BVMO crystal structure. After various unsuccessful efforts with other BVMOs in the past, Mattevi and co-workers managed to solve the crystal structure of PAMO (Malito et al., 2004). The structure showed that the monooxygenase exhibits a two-domain architecture with FAD- and NADPH-binding domains and the active site located in a cleft at the domain interface (Figure 3). Remarkably, the conserved BVMO sequence motif was found to be far from the active site, forming a surface loop that connects the FAD and NADP domains. The active site contains a strictly conserved arginine (R337 in PAMO), which was suggested to play a role in the stabilisation of the peroxyflavin intermediate (Figure 2B). However, a detailed kinetic study provided evidence that this intermediate is still formed and stabilised upon replacing R337 by an alanine (Torres Pazmiño et al., 2008).

Mirza et al. reported on two NADP-complexed crystal structures of CHMO\textsubscript{RhodoH1-31} which revealed two different conformational states (Mirza et al., 2009). These findings clearly confirm the structural dynamics that was predicted to occur during catalysis. The observed “sliding” of the coenzyme appears to involve multiple loop movements and includes repositioning of the conserved active-site arginine (Figure 3). These movements are proposed to be coordinated by the previously mentioned BVMO-motif, providing an explanation for the conservation of this sequence motif.

Orru et al. solved in total 8 structures of wild-type PAMO and the mutants (R337K, D66A, and M446G, Orru et al., 2011). All but one of the new structures contained the NADP\textsuperscript{+} coenzyme. Furthermore, the authors showed that PAMO crystals are redox reactive, and by collecting the diffraction data on dithionite-treated crystals, they obtained structures of wild-type PAMO and the R337K mutant in reduced state. Importantly, in three of the solved structures, a molecule of 2-(N-morpholino)ethanesulfonic acid (MES) was found, and it was suggested to occupy substrate binding sites. This comprehensive work uncovered details of the interplay between R337, NADP\textsuperscript{+}, and FAD in oxygenation of the substrate. In the oxidised form of PAMO, R337 is involved in an H-bond with the carboxamide group of NADP\textsuperscript{+} and with the side chain of conserved D66 (Figure 4A). The latter residue plays important role in NADPH oxidation: it was proposed to position NADPH correctly in the active site and to facilitate NADPH oxidation by forming a favourable interaction with the positively charged NADP\textsuperscript{+}. Upon flavin reduction, R337 moves away from NADP\textsuperscript{+} towards the flavin and creates electrostatic interaction with the negatively charged reduced flavin (Figure 4B). At the same time, the rotation of the NADP\textsuperscript{+} carboxamide is observed, and the carboxamide
Figure 4. Snapshots of the catalytic cycle of PAMO. NADP\(^+\) is presented in blue, FAD in yellow, D66 and R337 in green. A. Active site of PAMO in the oxidised state (2YLR). B. Active site of dithionite-reduced PAMO (2YLS). C. PAMO in the oxidised state with MES (in purple) bound in the active site (2YLT).

Figure 5. A. Overlay of the structures of PAMO (2YLR, in dark blue), STMO (4AOS, in purple), CHMO\(_{\text{Rhodo} \text{HI-31}}\) in an open form (3GWF, in green) and OTEMO (3UOY, chain A, in cyan). FAD and NADP\(^+\) are shown in sticks. Different conformations of NADP\(^+\) adopted in different structures can be observed. B. Dimeric structure of OTEMO. The FAD-binding domain is presented in green, the NADP\(^+\)-binding domain in cyan, and the flap domain in purple. C. Overlay of active sites of PAMO (in dark blue) and OTEMO (in cyan). FAD, NADP\(^+\), and catalytically relevant aspartate and arginine residues (D59 and R337 in OTEMO, D66 and R337 in PAMO) are shown in stick representation.

group establishes an H-bond with N5 of the flavin. This H-bonding prevents the reduced flavin from losing its N5 proton. This interaction is assumed to have a stabilising effect on the peroxyflavin. After the peroxyflavin is formed, R337 moves back creating space for the substrate and can interact with the substrate carbonyl oxygen in order to facilitate the nucleophilic attack by the peroxyflavin on the
carbonyl carbon. The structure of the PAMO-NADP⁺-MES complex (Figure 4C) enabled modelling of the Criegee enzyme intermediate. The model disclosed that R337 and NADP⁺-ribose participate in an H-bond interaction with the Criegee intermediate. This hints to a crucial role of R337 and NADP⁺ in stabilising this tetrahedral intermediate. These data demonstrate how the protein is able to form and stabilise peroxylavlin and perform substrate oxygenation by using the network of fine interactions between the active-site residues, the flavin, and NADP⁺.

Further insights into the catalytic mechanism of BVMOs are brought by the structural analysis of STMO (Franceschini et al., 2012). In the structure of this enzyme, NADP⁺ appears in another position than in PAMO, OTEMO, and CHMO<sub>RhodoH</sub><sup>31</sup>: it is shifted away from the flavin (Figure 5A). This binding mode of NADP⁺ was suggested to represent the position of the reduced coenzyme before the flavin reduction takes place and/or of the oxidised coenzyme before it leaves the active site. In order to support the dual function of the nicotinamide coenzyme in BVMOs (acting as an electron donor and as a structural element of the active site participating in stabilisation of crucial enzyme intermediates), it must move within the active site. This is in line with the “coenzyme sliding” model proposed for CHMO<sub>RhodoH</sub><sup>31</sup> (Mirza et al., 2009).

OTEMO is the first dimeric BVMO that was crystallised (Leisch et al., 2012). The structure (Figure 5B) reveals that the dimer is formed by contacts between helices from NADP-binding and flap domains. Similarly to PAMO, the interactions between key active-site elements: FAD, NADP⁺, R337, and D59 can be observed (Figure 5C). In the structure of OTEMO, R337 points away from NADP⁺ and makes a contact with the flavin. In turn, NADP⁺ interacts with the N5 atom of the FAD isoalloxazine ring. However, the conformation of D59 is different than that in PAMO and allows H-bonding with R337. The roles of Y53, D59, and R337 in OTEMO (corresponding to Y60, D66, and R337 in PAMO) were tested. The Y53F mutant displayed significant activity while the Y53A protein was insoluble. Other mutants (D59A, D59N, R337A, R337K), although soluble and containing FAD, showed no or very little activity which proved the essential role of these residues also in OTEMO. Furthermore, four distinct crystal forms of OTEMO were obtained which were characterised by different arrangements of several regions close to the active site (145–152, 390–394, 497–518). The apparent flexibility of these structural elements was suggested to be important for the acceptance of unusually big substrates, like CoA esters. However, as the authors admit, the actual substrate binding site of OTEMO is not yet well defined.
Substrate acceptance plasticity of BVMOs

An inventory of the substrate acceptance profiles of the newly cloned and studied BVMOs has indicated that each BVMO has a preference for a certain class of compounds. Typically, a BVMO-substrate class can be only roughly defined by size and type of molecules. For example, PAMO has evolved to convert a large set of aromatic compounds while it hardly accepts aliphatic compounds (Fraaije et al., 2005). CHMO\textsubscript{Acineto} readily accepts a wide range of bulky cyclic aliphatic ketones but does not convert aromatic or simple aliphatic compounds (Mihovilovic et al., 2002). The restriction in substrate scope is still not well understood. For instance, the protein sequence of PAMO is very similar to that of STMO (> 50% sequence identity) while it is not active on steroids. Comparison of the crystal structures of PAMO and STMO does not provide clear clues on which residues are responsible for the substrate discrimination. The ability of BVMOs to adapt in such an apparently subtle way to different substrate classes is probably a reason for widely disparate roles in metabolic pathways. BVMOs are used to degrade relatively small molecules, for example, an acetone-specific BVMO has been found in a Gordonia species (Kotani et al., 2007), while other BVMOs act on highly complex biomolecules like aflatoxin derivatives (Wen et al., 2005). Strikingly, no BVMO has yet been identified that exploits \textit{in vivo} the ability of BVMOs to perform other types of oxidation, such as sulfoxidation.

Redesign of BVMO for biocatalytic applications

Various attempts have been made to randomly and rationally redesign BVMOs. Several studies have been reported in which error-prone PCR was used to generate mutant libraries. Directed evolution of BmoF1 yielded various mutants with an enhanced enantioselectivity towards long-chain ketones (Kirschner and Bornscheuer, 2006; Kirschner and Bornscheuer, 2008). Based on a homology model, these altered residues appear to be far from the proposed active site. Also, CHMO\textsubscript{Acineto} and CPMO have been targeted by (semi-)random mutagenesis yielding several mutants with improved catalytic properties (Clouthier et al., 2006; Kayser and Clouthier, 2006; Mihovilovic et al., 2006). Some of the uncovered mutation sites appear to be in or near the active site. A more directed approach of enzyme redesign was performed on the thermostable PAMO. As this enzyme mainly acts on aromatic compounds, it has been attempted to broaden its substrate specificity to bulky, non-aromatic ketones. In the first redesign study, an extended bulge was identified in the structure of PAMO which is absent in CHMO\textsubscript{Acineto} (see insets Figure 3). Removal of two residues in this loop region yielded a variant that accepts the bulkier 2-phenylcyclohexanone as a substrate (Bocola et al., 2005). A more extended mutagenesis study of the bulge region (residues 441–444) yielded
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mutants that were able to enantioselectively convert 2-phenylcyclohexanone and a derivative (Reetz and Wu, 2008). Alternatively, a mutation of M446 to a glycine resulted in a mutant enzyme with the altered substrate specificity (for instance, formation of indigo blue from indole), improved enantioselective behaviour, and altered regioselectivity (Ríoz-Martínez et al., 2009; Torres Pazmiño et al., 2007). In an interesting redesign study by the Reetz group, two conserved prolines in the bulge region were targeted (Reetz and Wu, 2009). Somewhat unexpectedly, this revealed that replacement of P440 by a leucine or a phenylalanine renders “CHMO-like” mutants that are able to (enantioselectively) convert 2-substituted cyclohexanone derivatives. In another study, Reetz and co-workers demonstrated that targeting second shell residues can bring effects on the catalytic properties of the enzyme. By using site-saturation mutagenesis, they identified the Q93N/P94D mutant being active on 2-ethylcyclohexanone. This mutant was tested with several 2- and 4-substituted cyclohexanones and in most cases presented good to excellent enantioselectivity (Wu et al., 2010). Taken together, the PAMO redesign studies indicate that one loop region can play a dominant role in substrate discrimination while also second shell residues influence substrate acceptance of PAMO. Furthermore, it is worth noting that all reported PAMO mutants were thermostable which underlines that this specific BVMO and its derivatives are ideally suited for biocatalytic applications.

Saß et al. screened libraries in which several sites in 4-hydroxyacetophenone monooxygenase (HAPMO) from P. putida JD1 were saturated: G213, P214, Y537, G538, and T548, which are counterparts to Q93, P94, A435, G436, and N445 in PAMO (Saß et al., 2012). Two mutants T548S and T548C with increased specific activity with p-nitroacetophenone were identified. The T548 residue corresponds to N445 in PAMO which was not targeted previously. Yet, mutations in this region were confirmed to influence the substrate scope and selectivity of PAMO.

While PAMO remains the only thermostable BVMO available, efforts have been invested in increasing the stability of other BVMOs. Effects of removing cysteine and methionine residues on oxidative stability and thermostability of CHMO<sub>Acinetobacter</sub> have been investigated by Opperman and Reetz (2010). 12 methionines and 5 cysteines were replaced by hydrophobic residues (where possible, counterparts from PAMO or CHMO<sub>Rhodohalobacter</sub>: isoleucines, leucines, alanines, and valines. Mutants presenting increased residual activity after incubation with hydrogen peroxide or at elevated temperatures were isolated. A combination of stabilising mutations in MUT15 (8 mutations) and MUT16 (7 mutations) resulted in greatly improved oxidative stability and increased thermostability while the substrate acceptance and enantioselectivity were not affected.

Another approach of exploiting the (thermo)stability of PAMO was used by van Beek et al. (2012). A series of chimeric BVMOs was prepared by exchanging
the C-terminal part of PAMO (~100 residues) by homologous pieces from other BVMOs (STMO, CHMO\textsubscript{Acinetobacter}, and a putative BVMO gene from a metagenomic library). The resulting hybrid enzymes showed improved thermostability compared to the respective parent enzymes CHMO\textsubscript{Acinetobacter} and STMO while they present novel biocatalytic properties. These chimeric enzymes catalyse reactions not performed by PAMO or present higher enantioselectivity than both parent enzymes. These results prove that PAMO is a robust scaffold for creating BVMO chimeras with new catalytic properties.

Codexis Inc. engineered CHMO\textsubscript{Acinetobacter} for sulfoxidation of pyrmetazole to esomprazole, a drug used in treatment of several gastric diseases (Bong et al., 2011). The activity of CHMO\textsubscript{Acinetobacter} in this reaction was improved up to 10,000-fold, and the enantioselectivity was changed from > 95% ee for the (R)-enantiomer in the first rounds of evolution to almost 100% ee for the (S)-enantiomer. Furthermore, formation of a sulfone by-product was minimised, and the thermostability, tolerance to organic solvents as well as the expression of the enzyme were improved. This example shows that remarkable changes in properties of BVMOs can be achieved by protein engineering, but it also clearly indicates that such great improvements of naturally occurring enzymes are often necessary in order to establish economically viable processes.

**Applications of BVMOs in organic synthesis**

Rioz-Martínez et al. employed PAMO and HAPMO in dynamic kinetic resolutions of β-ketoesters (Figure 6A, Rioz-Martínez et al., 2011). The B–V oxidation of the racemic starting compound yielded enantiopure diesters. The racemisation of β-ketoesters was achieved by applying basic conditions (pH 9). Subsequently, the diesters were treated with corresponding alcohols under acidic conditions which allowed specific hydrolysis and led to enantiopure α-hydroxyesters. The process was applied for aliphatic and aromatic β-ketoesters yielding complete enantiopurity and good conversions. The B–V oxidation by HAPMO in combination with racemisation by exchange resins allowed a dynamic kinetic resolution of several benzylketones (Rodríguez et al., 2010). Different types of anion exchange resin were evaluated, and the best results were obtained with a weak resin: Dowex MWA-1. Moderate to good conversions and ee values were achieved. The same methodology was used for a dynamic kinetic resolution of benzylketones catalysed by the M446G mutant of PAMO in the presence of 5% methanol (de Gonzalo et al., 2012). Furthermore, the unusual regioselectivity of the M446G mutant of PAMO was exploited in a dynamic kinetic resolution of 2-alkyl-1-indanones which led to chiral 3-alkyl-3,4-dihydroisocoumarins. The racemisation was again
Figure 6. Selected oxidations catalysed by BVMOs. A. Dynamic kinetic resolution of β-ketoesters leading to enantiopure β-hydroxyesters. B. Production of chiral β-amino acids and β-aminoalcohols through regioselective oxidation of β-aminoketones. C. Kinetic resolution of organoboron compounds catalysed by PAMO. D. Oxidation of organoselenium acetophenones to selenoxides. E. Kinetic resolution of organoselenium compounds. F. Production of enantiopure aroma lactones by kinetic resolution of substituted cyclic ketones.
achieved by applying basic conditions, and the yield or enantiomeric excess could be manipulated by the addition of organic solvents (Rioz-Martínez et al., 2010).

In the work of Rehdorf et al., BVMOs were used to establish an enzymatic route to enantiopure \( \beta \)-amino compounds (Figure 6B, Rehdorf et al., 2010a; Rehdorf et al., 2010b). B–V oxidation of \( N \)-protected \( \beta \)-amino ketones led to \( \beta \)-amino esters. Due to the unusual regioselectivity of some enzymes, in several cases both regioisomeric lactones could be obtained. Subsequent hydrolysis yielded \( \beta \)-amino acids and \( \beta \)-amino alcohols. A panel of BVMOs were tested with several substrates leading to enantiocomplementary products with high optical purity.

Heteroatom oxidations by BVMOs have also been studied in the last few years. For instance, HAPMO, wild-type PAMO, and its M446 mutant were successfully employed in the preparation of chiral sulfoxides including heteroaryl, cyclohexyl, alkyl, and cyclic compounds (Rioz-Martínez et al., 2010b). Also, CHMO engineered by Codexis Inc. for the oxidation of pyrmetazole (\textit{vide supra}) is a fine example of the use of a BVMO for an enantioselective sulfoxidation of a prochiral sulfide. Furthermore, several BVMOs were explored for their ability to oxidise aromatic or vinylic boron compounds (Brondani et al., 2011). A high degree of chemoselectivity was demonstrated as in most cases the enzymes catalysed preferentially the boron oxidation and not B–V oxidation or epoxidation. A kinetic resolution of boron-containing compounds catalysed by a BVMO was shown for the first time: chiral alcohols and chiral boron compounds were obtained with high enantiopurity (Figure 6C). Andrade et al. demonstrated the oxidation of organoselenium acetophenones to selenoxides with PAMO (Figure 6D, Andrade et al., 2011). Lastly, several BVMOs were evaluated in a kinetic resolution of aromatic selenides, and PAMO was shown to catalyse the resolution of phenylselenide compound with high enantioselectivity (Figure 6E, Brondani et al., 2012).

The Mihovilovic group employed BVMOs in the synthesis of optically pure aroma lactones (Figure 6F, Fink et al., 2011b). Initially, seven BVMOs were tested in a kinetic resolution of four racemic 2-substituted cyclic ketones. Two best-performing enzymes, CHMO from \textit{Arthrobacter} BP2 and cyclododecanone monooxygenase, were applied on a preparative scale resulting in production of enantiopure jasmine lactones and their \( \varepsilon \)-caprolactone homologs with good yields and excellent \( ee \) values (from 93% \( ee \) to \( > 99\% \) \( ee \)).

Comprehensive substrate profiling of cyclopentadecanone monooxygenase (CPDMO) from \textit{Pseudomonas} sp. strain HI-70 revealed some unprecedented oxidations catalysed by this enzyme (Fink et al., 2011a). As an example, CPDMO was able to convert (−)-menthone. Moreover, CPDMO provided access to the \((S,S)\)-enantiomer of the Geissman–Waiss lactone, a precursor of necine alkaloids.
Coenzyme regeneration for BVMO-based processes

As BVMOs require an electron donor (typically NADPH) for activity, BVMO-based biocatalytic applications have to deal with this in a cost-effective manner. In numerous studies, it has been established for recombinant cells expressing BVMOs that NADPH can be regenerated by making use of the coenzyme recycling system of the host (for examples see (Berezina et al., 2007; Cernuchova and Mihovilovic, 2007; Mihovilovic et al., 2008a; Mihovilovic et al., 2008b; Rial et al., 2008a; Rial et al., 2008b; Yang et al., 2009)). In combination with in situ substrate feeding and product removal, biotransformations yielding up to 1 kilogram of B–V product can be performed (Hilker et al., 2008). Successful attempts to improve the efficiency of the B–V reaction in recombinant cells include the coexpression of glucose-6-phosphate dehydrogenase in E. coli (Lee et al., 2007), coexpression of NADH kinase (Lee et al., 2012), and application of Corynebacterium glutamicum as an expression host (Doo et al., 2009). The latter organism is known to possess high coenzyme regeneration capacity.

Recently, we have shown that by fusion engineering the catalytic efficiency of E. coli expressing CHMO\textsubscript{Acinetobacter} and CPMO could be increased. In addition, by fusing several BVMOs to phosphite dehydrogenase (PTDH), effective B–V oxidations could be performed using cell-free systems (Torres Pazmiño et al., 2008). The efficiency of the self-sufficient BVMO system was further improved by (1) addition of an affinity purification tag, (2) substitution of the dehydrogenase subunit by a more thermostable PTDH, and (3) expanded to other members of the Type I BVMO family (Torres Pazmiño et al., 2009).

Gotor et al. developed a PIKAT (parallel interconnected kinetic asymmetric transformations) system, in which reactions catalysed by a BVMO and an alcohol dehydrogenase (ADH) occur simultaneously and are connected via NADP(H) as a shared coenzyme (Figure 7, Bisogno et al., 2010; Rioz-Martínez et al., 2010a). By using a double kinetic resolution realised by PAMO, the PAMO mutant M446G, or HAMO, and ADHs from Lactobacillus brevis or Thermoanaerobacter sp., they achieved enantiopure aromatic ketones and sec-alcohols. When a kinetic resolution of sec-alcohols catalysed by ADHs was combined with desymmetrisation of sulfides by PAMO or HAMO, sulfoxides were obtained with high optical purity.

Another elegant approach to circumvent the need for coenzyme regeneration is the application of (sun)light and a suitable sacrificial electron donor (Hollmann et al., 2007). The work of Hollmann and colleagues has demonstrated that light, with the help of effective mediators, can be used to reduce the flavin cofactor in BVMOs. This is an attractive approach albeit that the reported efficiency was very poor. Drastic improvements on this system are needed to enable effective light-driven BVMO-mediated biocatalysis (Taglieber et al., 2008).
Conclusions

The last few years have seen interesting progress in the BVMO-related studies (Figure 1). This has revealed that BVMOs are abundantly present in nature and are responsible for widely varying tasks. The biodiversity has been exploited by classical cloning strategies, but also genome mining efforts have led to a formidable increase in the number of available BVMOs. Biocatalytic studies have shown that BVMOs can be utilised for a startling number of different substrates, while often exhibiting exquisite regio- and/or enantioselectivity. Another scientific breakthrough is the elucidation of BVMO structures which has led to a better understanding of how these enzymes function. However, some details on how separate catalytic events take place remain unclear. For example, what conformational changes occur during a full catalytic cycle? Another challenge is solving the structure of the peroxylavin intermediate. Moreover, a good view on the substrate binding pocket is lacking and clearly asks for more structural studies on this class of oxidative enzymes. This would enable more directed enzyme-redesign approaches for fine tuning BVMO towards biocatalytic applications. Except for the discovery or redesign of suitable BVMOs, challenges still remain in developing cost-effective technical approaches to apply BVMOs which should enable effective oxygen supply and coenzyme recycling.

Figure 7. Application of the PIKAT method for the preparation of enantioenriched (A) ketones and sec-alcohols and (B) sulfoxides and sec-alcohols.
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References


Baeyer–Villiger monooxygenases


Baeyer–Villiger monooxygenases


