

Baeyer–Villiger Monooxygenases: More Than Just Green Chemistry

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1. INTRODUCTION

What is life without oxygen is a rhetorical question. On the other hand, unraveling the intricacies and understanding the various mechanisms underpinning the biological processes beg many answers. The activation of hydrocarbon C–H bonds by oxygenases exemplifies an important biological process and prerequisite to the eventual transformation of these raw materials into value-added chemicals or bioproducts. Oxygenases come in two forms: those that introduce one atom of molecular oxygen into an organic substrate, called monooxygenases (also referred to as mixed function oxygenases) and those that insert both oxygen atoms into a substrate, namely, dioxygenases. For a historical account on the discovery of oxygenases see a review by Hayaishi.¹ In monooxygenase-catalyzed reactions, the other oxygen atom undergoes reduction to water. Hence, in a biotransformation or biocatalysis setting, having water as a byproduct cannot be greener. This review focuses on the monooxygenase-catalyzed Baeyer–Villiger oxidation of linear or cyclic ketones as a green chemistry tool to address environmental sustainability, a system to study its molecular diversity and catalytic mechanism, industrial-scale bioprocess development, and a challenging model for protein engineering to evolve new biotechnological applications.

Biocatalysis at large is poised to play an ever increasingly important role in meeting the needs of industrial and environmental sustainability as manufacturers and industries are striving to improve efficiency and implement cleaner processes.² In the context of the three pillars of sustainable development, the use of biocatalysts, as opposed to strictly harsh chemical methods, is meeting the needs of environmental care and social responsibility, although rapid economic progress requires serious financial investment.

2. THE CHEMICAL BAEYER–VILLIGER REACTION

In 1899 at the age of 64, Adolf von Baeyer (previously known for the synthesis of indigo and a Nobel laureate in chemistry in 1905) and his student Victor Villiger reported on the use of potassium monopersulfate (KHSO₅; also known as Caro's acid) as a new oxidant for the conversion of cyclic ketones such as menthone (**1**), carvomenthone, and camphor to the corresponding lactones with yields up to 50% in a reaction carried out at room temperature for 24 h (Figure 1).³ The transformation of ketones into esters or cyclic ketones into lactones by peracids became the famous Baeyer–Villiger (BV) oxidation in synthetic organic chemistry. An earlier review by Krow highlights the wide-ranging applications of this transformation, from the synthesis of steroids, antibiotics, and pheromones to the synthesis of monomers for polymerization.⁴ Now almost a century and a dozen years later, the development of the BV reaction is still going strong marked by quests for oxidants that are more chemoselective and efficient,⁵ catalysis with transition

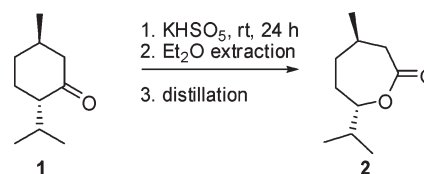


Figure 1. Oxidation of menthone by Baeyer and Villiger.

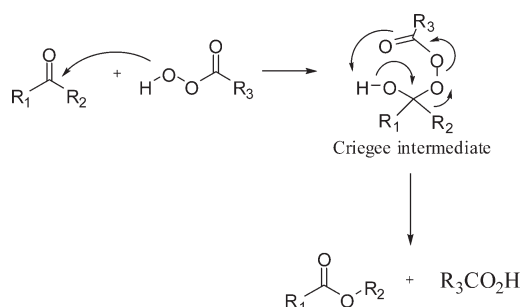


Figure 2. Proposed mechanism for the BV oxidation by Criegee.

metal complexes, and also the use of ionic liquids.⁶ On the chemical aspects of BV oxidations, readers are referred to several key recent reviews.⁷

The Criegee mechanism underscores the essence of the BV reaction in two steps (Figure 2).⁸ First, there is the nucleophilic attack or addition of the peroxy acid to the carbonyl to form a tetrahedral intermediate, otherwise known as the Criegee intermediate. Then, a concerted migration of one of the adjacent carbons to oxygen leads to the release of the carboxylate anion or carboxylic acid. The second step is usually rate-limiting with the leaving group playing a critical role. If the migrating carbon is chiral, the stereoselectivity is retained. The latter is the foundation of one of the advantages of BV oxidations. Other advantages include a variety of carbonyl substrates that can be oxidized, tolerance to a large number of functional groups that can be present on the molecules to be oxidized, a relatively wide choice of oxidants, stable or otherwise, and also, regioselectivity that is predictable on the basis of the migrating group. Interestingly, despite the fact that the Criegee mechanism was described some 60 years ago, the reaction mechanism is still investigated.⁹

Despite the above-mentioned advantages, the classical BV oxidation lacks the high chemo-, regio-, and enantioselectivity and broad substrate specificity that are needed or expected for organic synthesis. Equally if not more important is the waste associated with the use of an organic peracid; one equivalent of the corresponding carboxylic acid salt is produced, which needs to be disposed of or recycled. In addition, many of the chemical oxidants (e.g., *m*-chloroperoxybenzoic acid, hydrogen peroxide in combination with a Lewis acid) are either intrinsically unstable, costly, shock sensitive, or explosive in condensed form. These shortcomings of safety considerations and other limitations to applications are addressed and met to a large extent by the biological BV reactions as described below.¹⁰

3. BIOLOGICAL BAEYER–VILLIGER REACTIONS

3.1. Occurrence and Classification

It is generally believed that in 1948 Turfitt provided the first indication of the existence of a biological BV reaction in a series of

Table 1. General Characteristics and Classification of BVMOs

type 1 (I) BVMO, class B FMO	type 2 (II) BVMO, class C FMO	type “O” BVMO, class A FMO
single gene product	two separate gene products, reductase and oxygenase	single gene product
contain a tightly bound FAD cofactor	use reduced FMN generated by the reductase as a coenzyme	FAD bound to the surface of protein
NADPH-dependent	the reductase can use NAD(P)H	NAD(P)H-dependent
NADPH/NADP ⁺ bound during catalysis		
“BVMO” fingerprint motif sequence: FXGXXXHXXXWP	absence of “BVMO” fingerprint	absence of “BVMO” fingerprint
two domain structures resembling disulfide oxidoreductases; two dinucleotide-binding domains (Rossmann fold)	structural core of the oxygenase subunit displays a TIM-barrel fold	structural relationship to FAD-dependent hydroxylases
binding FAD and NADPH		

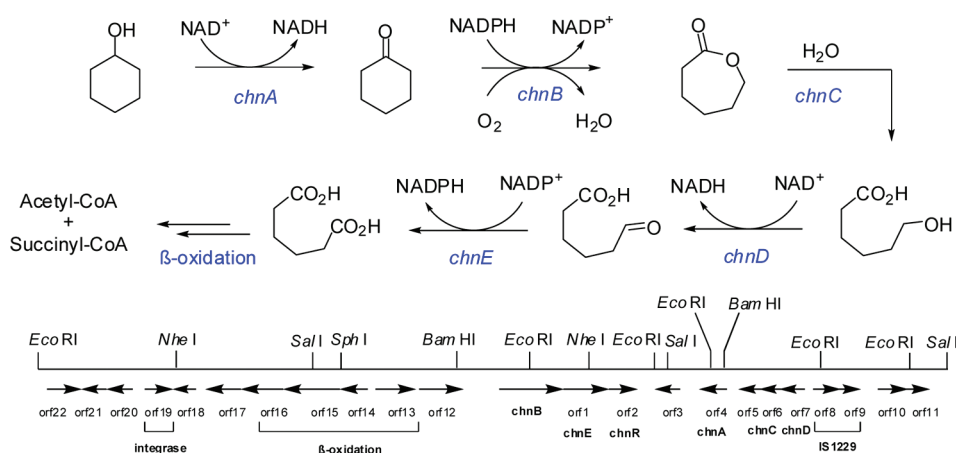


Figure 3. Biochemical steps of cyclohexanol degradation and the responsible genes in *Acinetobacter* sp. NCIMB 9871. The directions of the arrows indicate divergent or convergent transcription of the respective genes or open reading frames (orfs). The encoded enzymes are as follows: ChnA, cyclohexanol dehydrogenase; ChnB, cyclohexanone 1,2-monooxygenase (CHMO); ChnC, caprolactone hydrolase; ChnD, 6-hydroxyhexanoate dehydrogenase; ChnE, 6-oxohexanoate dehydrogenase; ChnR, transcriptional activator. Further oxidation of adipic acid to acetyl-CoA and succinyl-CoA is presumed to proceed via β -oxidation.²²

microbiological investigations of steroids.¹¹ However, the conversion of progesterone to testolactone with some 70% yield by fungi such as *Penicillium chrysogenum* or *Cylindrocarpum radicola* provided the first definitive evidence of lactone formation, namely in the D-ring.¹² Testosterone was similarly oxidized to estrolactone. The first Baeyer–Villiger monooxygenase (BVMO) (E.C. 1.14.13.x) to be purified to homogeneity and shown to be a flavoprotein with a broad ketone substrate specificity was provided by studies of Trudgill and co-workers on the metabolism of simple alicyclic alcohols such as cyclohexanol¹³ and cyclopentanol¹⁴ and their corresponding ketones.¹⁵ Earlier, Gunsalus and co-workers working on the bicyclic monoterpene, (+)-camphor, had partially purified two enzymes from a fluorescent pseudomonad C₁ strain that were involved in the lactonization of camphor.¹⁶ Referred to as a flavin mononucleotide (FMN)-coupled diphosphopyridine nucleotide (DPNH) oxidase (E_1) and a ketolactonase (E_2), this coupled enzyme system is a prototype of what we know nowadays as a member of the type 2 BVMOs based on the available N-terminal amino acids of the respective polypeptides.¹⁷ Type 2 BVMOs are composed of two components, a reductase and an oxygenase, the former utilizes NADH to reduce FMN while the latter uses the reduced flavin to perform the BV reaction. The prototype BVMOs from the alicyclic alcohol/ketone pathways, namely, the cyclohexanone monooxygenase

(CHMO; EC 1.14.13.22) from *Acinetobacter* sp. NCIMB 9871 and cyclopentanone monooxygenase (CPMO; EC 1.14.13.16) from *Comamonas* (previously, *Pseudomonas*) sp. NCIMB 9872,¹⁸ are among the first members of the type 1 BVMOs.¹⁷

The present classification of BVMOs in the family of flavoprotein (flavin-containing) monooxygenases (FMOs) at large, places type 1 BVMO in the class B FMOs and those of type 2 in the class C FMOs.¹⁹ A new type “O” BVMO classification (belonging to class A FMO)¹⁹ represented by MtmOIV, known as an “atypical” BVMO, otherwise “odd”, appears justifiable.²⁰ Sequence-wise and structurally, the latter is neither that of type 1 nor type 2. General characteristics of the respective classes are shown in Table 1, and additional biochemical details can be found in later sections.

3.2. Function and Significance

As many oxygenases are, the presently known BVMOs are largely found in catabolic pathways, the prototypes being the degradation of cyclohexanol and cyclopentanol and their corresponding ketones.^{13,18,21} Microorganisms capable of growth on cycloalkanol or cycloalkanone metabolize the respective substrate to intermediates such as dicarboxylic acids (glutaric acid in the case of C₅ and adipic acid in the case of C₆ compounds) that are further oxidized to acetyl-coenzyme A via the fatty acid β -oxidation pathway as shown in Figure 3. Thus, it may come as no surprise that in the prototypical *Acinetobacter* sp. strain

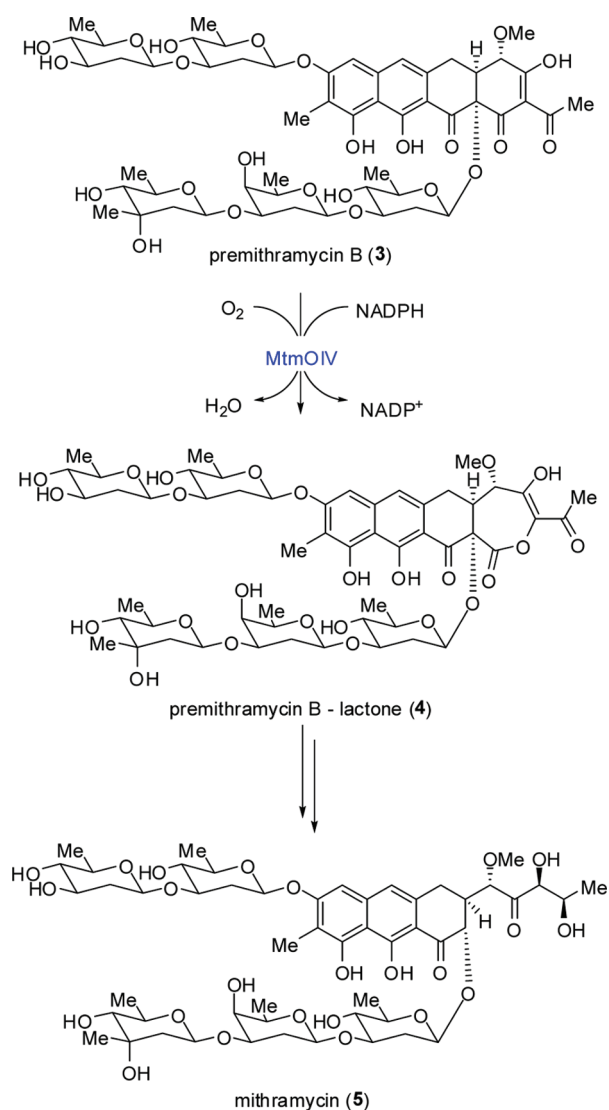


Figure 4. Enzymatic Baeyer–Villiger oxidation in the biosynthesis of mithramycin (5).

NCIMB 9871, potential β -oxidation pathway genes were found associated with the catabolic genes of cyclohexanol (Figure 3).²²

The BVMO reaction represents the second step in the degradation pathway after the initial oxidation of cyclohexanol to cyclohexanone by an NAD-dependent cyclohexanol dehydrogenase. This is also true for the catabolism of cyclopentanol by *Comamonas* sp. NCIMB 9872¹⁸ or larger ring compounds such as cyclododecanol and cyclopentadecanol.²³ Metabolism of cyclohexylamine by *Brevibacterium oxydans* IH-35A also resulted first in the production of cyclohexanone via the action of cyclohexylamine oxidase, which was followed by BVMO oxidation.²⁴ Lactonization of a cycloalkanone by a BVMO prepares the ring-expanded molecule for hydrolysis by ring-opening enzymes such as ϵ -caprolactone hydrolase or 5-valerolactone hydrolase in the degradative pathways of cyclohexanol and cyclopentanol, respectively. Hydrolytic ring opening of a lactone after ring expansion by BVMO oxidation is analogous to the initial steps of bacterial metabolism of aromatic hydrocarbons where ring cleavage occurs after the first obligatory ring modification by mono- or dioxygenation.²⁵

In the biosynthetic pathway of an anticancer antibiotic called mithramycin by the soil bacterium *Streptomyces argillaceus* ATCC 12956, a BVMO named MtmOIV was found to be responsible for the conversion of a biologically inactive precursor, premithramycin B (3) to the corresponding lactone 4 that is further converted to mithramycin DK presumably by lactone opening and decarboxylation, and finally into the active drug mithramycin (5).^{20,26} MtmOIV is believed to catalyze more than the fourth ring modification of premithramycin but also the various follow-up reaction steps.²⁰ MtmOIV is regarded as the first example of a naturally occurring BVMO with its cognate substrate (Figure 4).

Another BVMO with a specific biosynthetic role is that of PtlE derived from the biosynthesis pathway of a sesquiterpenoid antibiotic, pentalenolactone, found in the Gram-positive soil bacterium *Streptomyces avermitilis*.²⁷ PtlE is responsible for the conversion of 1-deoxy-11-oxopentalenic acid to neopentalenolactone D. The PtlE-encoding gene is among a large gene cluster that includes cytochrome P450 and non-heme iron/ α -ketoglutarate-dependent dioxygenase. PtlE is a bona fide type I BVMO, unlike MtmOIV. Described as representing a new branch of the pentalenolactone family tree, PtlE members includes the cyclopentadecanone monooxygenase (CPDMO) of *Pseudomonas* sp. HI-70 that shows 52% sequence identity and a substrate specificity that includes C_6 to C_{15} alicyclic ketones.

Potential BVMO candidates have also been identified in important biosynthesis pathways such as the polyketide-derived secondary metabolites (aflatoxins) produced by *Aspergillus parasiticus*,²⁸ and in the biosynthesis of aurafurones and aurafuron-like structures in myxobacterial strain *Stigmatella aurantiaca* and *Streptomyces* sp.²⁹ Homologues of CPMO were reported in the loline alkaloid gene cluster identified in the fungal symbiont *Neotyphodium uncinatum*.³⁰ Unusual as MtmOIV may be, its homologues were found in the gene cluster of *Streptomyces aculeolatus* NRRL 18422 in the predicted biosynthetic pathway leading to the synthesis of 5-alkenyl-3,3(2H)-furanones such as E-837.³¹

3.3. Diversity of Baeyer–Villiger Monooxygenases

Table 2 is a list of BVMO-producing strains that are either in recombinant form or originating from natural isolates, largely from bacteria. Some biochemical properties of the enzymes are included together with the plasmids carrying the various cloned BVMO-encoding genes.

3.3.1. The Prototype *Acinetobacter* Cyclohexanone Monooxygenase. Cloning of a requisite BVMO-encoding gene in a heterologous host such as *Escherichia coli* (*E. coli*) is invariably necessary for at least three reasons. First, the natural host may be pathogenic or an opportunistic pathogen as in the case of *Acinetobacter* sp. NCIMB 9871, often referred to as a *calcoaceticus* species. The *Acinetobacter* genus, *A. baumannii* in particular, is emerging as one of the major nosocomial infectious pathogens facilitated by multidrug resistance.⁷² Second, a lactone ring-opening hydrolase gene is often associated with the BVMO-encoding sequence, and hence decoupling would be necessary to preserve the lactone structure. Third, the possibility of over-expression of the desirable BVMO is facilitated in addition to providing a better starting material for biotransformations especially if the natural producing strain is a slow or fastidious grower. Cloning of the first BVMO-encoding gene, that of the prototypical CHMO was pioneered by Walsh and co-workers.³² However, the correct 543-amino acid polypeptide sequence was established by Edman degradation and mass spectroscopy

Table 2. Native and cloned BVMOs^a

strain origin	common name	<i>E. coli</i> expression plasmid/strain or gene locus	optimum pH	number of amino acids	native molecular mass × 1000 Da	K_M (μ M)	specific activity (μ mol of substrate/(min/mg of protein))	ref
<i>Acinetobacter</i> sp. NCIMB 9871	CHMO _{Acinet}	native JM105pKK223–3 BL21(DE3)pMM4; JM109(pCM100); JM109(DE3)pET-22b TOP10[pQR239] <i>C. glutamicum</i> pEKEx2- <i>dmB</i>	9.0	543	59	6.9	22.6	13
<i>Acinetobacter</i> sp. SE19	CHMO	Cosmid clone		543				32
<i>Arthrobacter</i> sp. BP2	CHMO _{Athro}	pTrr-His-topo		591			3.57	33
<i>Arthrobacter</i> sp. L661	CHMO	BL21(DE3)pETCHMO-His	7.0	541			24.75	21b
<i>Brachymonas petroleovorans</i>	CHMO _{Brachy}	pTrr-His2-topo		537			0.4	33b
<i>Brevibacterium epidermidis</i> HCU	CHMO _{Brevi}	DH10BpPCB3		553			3.2 (<i>Brevi</i>), 2.0 (<i>Brevi</i> 2)	34
				529				35
<i>Comamonas</i> sp. NCIMB 9872	CPMO	native DH5 α (pCMP201) BL21(DE3)(pET22b(+)) BL21(pET11a)	7.7	550	200 (54–58 subunits)	<0.85	4.3 (cyclopentanone)	21c
		native		550				36
<i>Cylindrocarpum radiculicola</i> ATCC 11011	SMO	native	7.8	550	116 (dimer) 56 (monomer)	0.41	0.1 (cyclohexanone) progesterone	37
<i>Exophiala jeikei</i> KUF1-6N	CHMO	native	8	533	74			41
<i>Gordonia</i> sp. TY-5	ACMO	Rosetta(DE3)pEACMA B834 (DE3) pDB1 (Rv0892) B834 (DE3) pDB2 (Rv0565c) B834 (DE3) pDB3 (rv3854c), Top10(pBETAI)	8–8.5	495	230 (4 × 63)	0.48	0.68 (cyclohexanone) 0.61 (acetone)	42
<i>Mycobacterium tuberculosis</i> H37Rv	BVMO _{Myco}	B834 (DE3) pDB3 (rv3854c), Top10(pBETAI)		486				43
	EtaA	B834 (DE3) pDB3 (rv3854c), Top10(pBETAI)		489	56	61	k_{cat} 0.017 s ^{−1} phenylacetone	44
		B834 (DE3) pDB4 (Rv1393c) B834 (DE3) pDB5 (Rv3049c) B834 (DE3) pDB6 (Rv3083)		492				45
<i>Nocardia globetula</i> CL 1	CHMO	native	8.4	495	53	1.56	9.9	45,47
<i>Nocardia</i> sp. NCIMB 11399	CHMO	pSD80		524	83.95			45
<i>Pseudomonas aeruginosa</i> PAO1	CHMO	Rosetta Gami (DE3)pGEX-KG		527				unpublished
<i>P. fluorescens</i> ACB	HAPMO	native BL21(DE3)pLysS(pET-5a) pBAD/ <i>myc</i> -HisA vector JM109(pABE)		640	140 (2 × 70)	39	5.5 (4-hydroxyacetophenone)	48
<i>P. fluorescens</i> DSM 50106	BmoF1 (alkane)			640		9.2	k_{cat} 12.6 s ^{−1}	49
<i>P. putida</i> JD1	HAPMO _{JD1}	native		512	56			49,50
		RosettaET22b(+)/pJD14HAPMO	8	640	70	47	8.1 (4-hydroxyacetophenone)	51
<i>P. putida</i> KT2440	BVMO _{KT2440}	JM109pJOE-KT2440pGro7		508	106 (dimer) 56 (monomer)	38.1	23.2 (4-hydroxyacetophenone)	52
<i>P. putida</i> ATCC 17453	OTEMO, MO2	native	9.0				2.68 (2-oxo- Δ^3 -4,5,5-trimethyl-cyclopentenylacetyl)-CoA)	53
								54
								55

Table 2. Continued

strain origin	common name	<i>E. coli</i> expression plasmid/strain or gene locus	optimum pH	number of amino acids	native molecular mass × 1000 Da	K_M (μ M)	specific activity (μ mol of substrate/(min/mg of protein))	ref
<i>P. cepacia</i>	TDMO	native	7.8–8.0		123 (dimer) 55 (monomer)		0.49 (2-tridecanone)	56
<i>Pseudomonas</i> sp. HI-70	CPDMO	BL21(pCD201)	9	601	64	5.8	3.94 (cyclopentadecanone)	23b
<i>P. veronii</i> MEK700	MEKMO	BL21pAM262	9–10	549	60	6	1.12 (methylketone)	57,58
<i>Rhodococcus erythropolis</i> DCL 14	MMKMO	native	9.0		60	130	3.1 (4R-dihydrocarvone)	59
<i>R. jostii</i> RHA1	BVMO (MO 1–23)	Rosetta 2 (DE3)pLysS pET-YSBLJC-3C (MO 1–23)		364–663				60
<i>R. rhodocrous</i> IFO3338	SMO	native			60	100	1.12 (progesterone)	61
<i>R. ruber</i> SC1	CDMO	BL21(DE3)pSMO-EX pDCQ7 or pDCQ8		549	60	55	0.9 (progesterone)	62
<i>Rhodococcus</i> sp. HI-31	CHMO _{RhodHI-31}	pSDRmChnB1		603	65–70			23a
<i>Rhodococcus</i> sp. Phi 1	CHMO _{Rhodol}	pTrc-His-topo		540/560			6.0 (cyclohexanone)	63
<i>Rhodococcus</i> sp. Phi2	CHMO _{Rhodol2}	pTrc-His-topo		541			3.68	36
<i>Rhodococcus</i> sp. TK6	CHMO	BL21(DE3) pET21a(+) (pETCM-His)	7.5	540	60		3.75	36
<i>Streptomyces aculeolatus</i> NRRL18422	BVMO (ORF8HoxC)	gene cluster		504			226.3 (cyclohexanone)	64
<i>S. avermitilis</i>	PtE	BL21(DE3)pET31bMox		594	64			31
<i>S. coelicolor</i>	CHMO (MO 96) CHMO (MO 103)	Rosetta Gami (DE3)pGEX-KG		603	92.33 83.09			27 48
<i>Streptomyces</i> sp. Eco86	BVMO (ORF13HoxC)	gene cluster		550				31
<i>Thermobifida fusca</i> YX	PAMO	TOP10pPAMO	8.0	542	65	59	k_{cat} 1.9 s ^{−1} phenylacetone	65,66
<i>Xanthobacter</i> sp.	CHMO	native			50	<0.5	2.1 (cyclohexanone)	67
<i>Xanthobacter</i> sp. ZL5	CHMO _{Xantho}	BL21(pET11a)/ BL21(DE3)p11XS.1		546			~0.24 (cyclohexanone) ²⁴	41,68
<i>P. putida</i> ATCC 17453	2,5-DKCMO	native	Type II BVMO (Class C FMO)		78 (dimer) 37 (monomer)		3.56 (2,5-diketocamphane)	69
	2,5-DKCMO-1	pSD80						unpublished
	2,5-DKCMO-2	pSD80						unpublished
	3,6-DKCMO	native			76 (dimer) 40 (monomer)		0.244 (−)-camphor	70
		pSD80						unpublished
<i>S. aculeolatus</i> NRRL 18422	CHMO	gene cluster	Type O BVMO		504			31
<i>S. argillaceus</i>	MtmOIV	BL21(DE3)pLysS PRSETb Histag		570	56	34	premithramycin B	20,71

^a The majority are Type I BVMOs. Abbreviations: ACMO, acetone monooxygenase; CDMO, cyclohexanone monooxygenase; CHMO, cyclohexanone monooxygenase; CPDMO, cyclopentadecanone monooxygenase; CPMO, cyclopentanone monooxygenase; DKCMO, diketocamphane monooxygenase; EtaA, ethionamide monooxygenase; HAPMO, 4-hydroxyacetophenone monooxygenase; MEKMO, methylketone monooxygenase; MtmOIV, mithramycin monooxygenase; OTEMO, 2-oxo- Δ^3 -4,5-trimethylcyclopentynyl-acetylCoA monooxygenase; PAMO, phenylacetone monooxygenase; PtE, 1-deoxy-11-oxopentalenic acid monooxygenase; SMO, steroid monooxygenase; TDMO, 2-tridecanone monooxygenase.

sequencing of both the recombinant and native forms of CHMO from strain 9871,⁷³ following the resequencing and cloning of the same CHMO a decade after.^{21b} Subsequently, the complete cyclohexanol (*chn*) degradation gene cluster for the *Acinetobacter* sp. strain NCIMB 9871 was elucidated to consist of *chnBER* (*chnB*, cyclohexanone monooxygenase; *chnE*, 6-oxohexanoate dehydrogenase; *chnR*, transcriptional activator) organized in one operon, and *chnADC* (*chnA*, cyclohexanol dehydrogenase; *chnD*, 6-hydroxyhexanoate dehydrogenase; *chnC*, caprolactone hydrolase) that are divergently transcribed in another possible operon (Figure 3).²²

3.3.2. Dawn of a New Century of Cloned Baeyer–Villiger Monooxygenases. Recloning of the *Acinetobacter* CHMO-encoding gene and sequencing in 1999 marked the revival and a steady growing phase of newly cloned BVMOs. At the time of this writing, close to 60 clones of BVMOs derived from various microbial sources are available. Much of this was aided by the influx of completely sequenced microbial genomes that have now surpassed a thousand.⁷⁴ A notable genome endowed with putative BVMO sequences is that of the Gram-positive and pollutant-degrading *Rhodococcus jostii* strain RHA1 that yielded 23 candidates, all of which have been cloned although the expression level of some genes are suboptimal or not at all.⁶⁰ The biotransformation potential of the various cloned BVMOs is discussed in later sections.

Figure 5 shows a rooted phylogenetic analysis of the currently available cloned BVMO sequences. As expected, MtmOIV is an outlier together with a putative BVMO from *Streptomyces aculeolatus* NRRL 18422.⁵¹ Otherwise, three major branches subdivided into clusters of various sizes represent the diversity of the available BVMO sequences. Suffice it to say that the family clustering of BVMO sequences has been correlated to stereo-preferences of some BVMOs as reported by Mihovilovic and co-workers.⁷⁵ From the list of BVMO sequences (Figure 5), clusters of these enzymes active on cyclic ketones (e.g., CHMO, CPMO), linear ketones (e.g., ACMO, MKMO), aromatic ketones (e.g., PAMO), and aryl-aliphatic ketones (e.g., HAPMO) or large ring cyclic ketones (e.g., CDMO and CPDMO) are evident. However there is no hard rule that substrate specificity is dictated by the relatedness of the respective protein sequences as will be elaborated in section 4.

What is the possible number of BVMO sequences that can be retrieved from the expanding list of microbial genome database? Evidently, this is a moving target. The current National Centre for Biotechnology Information (NCBI) entry, available for “genomic BLAST”, registers 1347 bacterial and 78 archaeal genomes. Interestingly, one BVMO sequence (Htu_3838) of archaeal origin derived from a halophilic soil archaeon (*Haloterrigena turkmenica* DSM 5511) thus far appears. This sequence of 554 amino acids is 42% identical to that of the prototypical *Acinetobacter* CHMO. The fact that this putative gene is plasmid-encoded on pHTUR01 (out of five plasmids) implicates a possible lateral gene transfer event.

When the Concise Microbial Protein BLAST program of NCBI was used to search for homologues of *Acinetobacter* CHMO, PAMO, CPMO, HAPMO, and MtmOIV as representative BVMO sequences, some 967–1366 hits were found. In the case of *Acinetobacter* CHMO, 802/1018 hits were detected. These sequences were found in a diverse taxonomy range, from α , β , γ , and Δ proteobacteria to high GC + mycobacteria, *Rhodococci*, *Nocardia* and *Frankia* and even in mosses such as *Physcomitrella patens* subsp. *patens*. The closest homology of CHMO (62%; 536 amino acids) is that from an MTBE-

degrading bacterium *Polaromonas* sp. JS 666. CPMO sequence retrieved 843/1087 hits. The highest homologue of 53% sequence identity (544 amino acids) is that from *Parvibaculum lavamentivorans* DS-1, a degrader of linear aliphatic sulfonates. CPDMO retrieved 741 out of 967 hits with the closest homologue (59% identity; 603 amino acids) coming from *Phenyllobacterium zuchneum* HLK1. MtmOIV retrieved 957/1366 hits. Its closest homologue (46% identity; 494 amino acids) is from *Streptosporangium roseum*. HAPMO retrieved 904/1152 hits. The closest homologue (59% identity; 542 amino acids) is that from *Sorangium cellulosum* 56.

All in all, genome mining of established BVMO is retrieving sequences from diverse genera, not just in soil but in the marine environment as well. A recent example of a PAMO homologue was found in an algal symbiont called *Dinoroseobacter shibae* DFL12, for example.⁷⁶ A more complete picture of the available genomic BVMO sequences would require a formal bioinformatics study that is outside the scope of this review.

4. BAEYER–VILLIGER MONOOXYGENASES IN ORGANIC SYNTHESIS

This part of the review will focus on the application of microbes possessing BVMO activity, crude enzyme extracts containing BVMOs, purified enzymes, and recombinant enzymes expressed in either *E. coli* or yeast expression as biocatalysts in organic synthesis.

4.1. Baeyer–Villiger Monooxygenases in Steroid Transformations

4.1.1. Steroid Degradation by Fungal and Bacterial Baeyer–Villiger Monooxygenases. The availability and pharmacological properties made steroids an interesting research object for organic chemists in the early days of chemistry. Therefore not surprisingly the first indication of an enzymatic BV oxidation was noted during fungal degradation studies of steroids by Turfitt in 1948. He reported the isolation of minute amounts of Windaus's acid from the biodegradation of cholest-4-en-3-one derivative **6** by *P. erythropolis*.¹¹ The isolation of testolactone (**7**) and other D-ring oxidized steroids from the fermentation with progesterone (**8**), testosterone (**9**), and cortisolone (**10**) by penicillia and aspergilli species were reported by three independent research groups in 1953.^{12,78} Although the enzymes were never identified by the authors, they speculated that the biooxidation follows the same principle as the chemical oxidation using peracids, based on the fact that they could isolate considerable quantities of partially oxidized intermediates.¹² Large scale experiments with up to 30 L fungal cultures were conducted and confirmed that the metabolic degradation of steroids by fungal strains proceeds as follows: progesterone (**8**) to progesterone acetate (**11**) to testosterone (**9**) to androst-4-ene-3,7-dione (**12**), and finally to testolactone (**7**) (Figure 6).⁷⁹ Experiments by Rakhit and Singh with 17 α -deuterioprogesterone as starting material and extensive studies by Carlström confirmed the previously assumed pathway and mechanism of the microbial degradation of the side chain of steroids and D-ring lactonization.⁸⁰ These initial research efforts were mainly driven by the potential commercial value of chemoenzymatic routes to valuable steroidal drugs such as cortisone or estrone, but instead of the aspired C-11 hydroxylation, research teams identified multiple oxidative changes on the D-ring as described above.

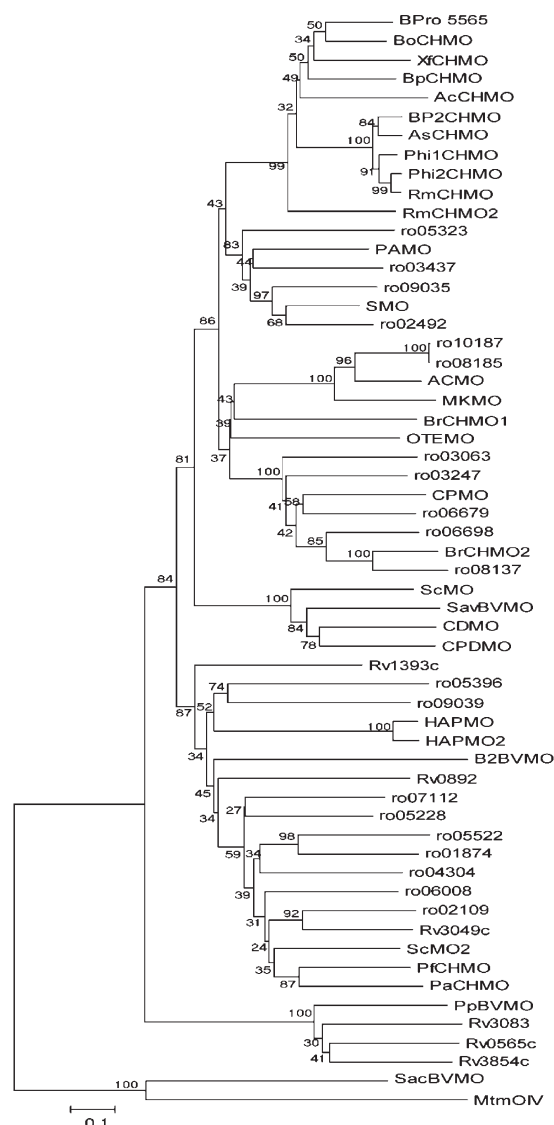


Figure 5. Phylogenetic analysis of the currently available cloned BVMO sequences. The BVMOs are listed with their species names as follows: AcCHMO, cyclohexanone monooxygenase from *Acinetobacter* sp. strain NCIMB 9871 (BAA86293.1, identical to AAG10021.1 of *Acinetobacter* sp. SE19, not shown); ACMO, acetone monooxygenase from *Gordonia* sp. TY-5 (BAF43791.1); AsCHMO, cyclohexanone monooxygenase from *Arthrobacter* sp. L661, (ABQ10653.1); B2BVMO, flavin monooxygenase from uncultured bacterium (ADE73876.1); BoCHMO, cyclohexanone monooxygenase from *Brevibacterium oxydans* IH-35A; BpCHMO, cyclohexanone monooxygenase from *Brachymonas petroleovorans* strain CHX (AAR99068.1); BP2CHMO, cyclohexanone monooxygenase from *Arthrobacter* sp. strain BP2 (AAN37479.1); BPro_5565, flavin-containing monooxygenase FMO from *Polaromonas* sp. JS666, (YP_552312.1); BrCHMO1 and BrCHMO2 (CHMO1 [AAG01289.1] and CHMO2 [AAG01290.1]), cyclohexanone monooxygenases from *Brevibacterium* sp. strain HCU, respectively; CDMO, cyclododecanone monooxygenase from *Rhodococcus ruber* strain SC1 (AAL14233.1); CPDMO, cyclopentadecanone monooxygenase from *Pseudomonas* sp. strain HI-70 (BAE93346.1); CPMO, cyclopentanone monooxygenase from *Comamonas* sp. strain NCIMB 9872 (BAC22652.1); HAPMO, 4-hydroxyacetophenone monooxygenase from *P. fluorescens* strain ACB (AAK54073.1); HAPMO2: 4-hydroxyacetophenone monooxygenase from *P. putida* JD1 (ACJ37423.1); MKMO, methyl ketone monooxygenase from *P. veronii* strain MEK700 (ABI15711.1); MtmOIV, mithramycin monooxygenase from *S. argillaceus* (3FMW_A); OTEMO, 2-oxo- Δ^3 -4,5,5-trimethylcyclopentenylacetic acid monooxygenase from *P. putida* ATCC 17453; PaCHMO, probable flavin-containing monooxygenase from *Pseudomonas aeruginosa* PAO1 (AE004582_5); PAMO, phenylacetone monooxygenase from *Thermobifida fusca* strain YX (AAZ55526.1); PfCHMO, putative flavin-binding monooxygenase from *P. fluorescens* strain DSM50106 (AAC36351.1); Phi1CHMO and Phi2CHMO, cyclohexanone monooxygenases from *Rhodococcus* sp. strain Phi1 (AAN37494.1) and *Rhodococcus* sp. strain Phi2 (AAN37491.1); PpBVMO, monooxygenase of flavin-binding family from *P. putida* KT2440, (AAN68413.1); RmCHMO, cyclohexanone monooxygenase from *Rhodococcus* HI-31 (BAH56677.1); SacBVMO, Baeyer–Villiger monooxygenase from *S. aculeolatus* (ABB88524.1); SavBVMO: putative monooxygenase from *Streptomyces avermitilis* MA-4680 (BAC70705.1); ScMO, putative monooxygenase from *S. coelicolor* A3(2), (CAB55657.1); ScMO2, putative monooxygenase from *S. coelicolor* A3(2), (CAB59668.1); SMO, steroid monooxygenase from *R. rhodochrous* strain IFO 3338 (AB010439.1); XfCHMO, *Xanthobacter flavus* strain ZL5 (CAD10801.1). The six Rv sequences are from the *Mycobacterium tuberculosis* H37Rv genome;⁴⁵ Rv3854c is a prodrug, ethionamide (EtaA) monooxygenase;⁴⁶ Rv3049c is BVMO_{mtb5}.⁴⁷ The 20 roXXXX are from the genome sequence of *R. jostii* RHA1.⁶⁰ The phylogenetic analysis was calculated using the program Muscle.⁷⁷ The scale 0.1 is the genetic distance.

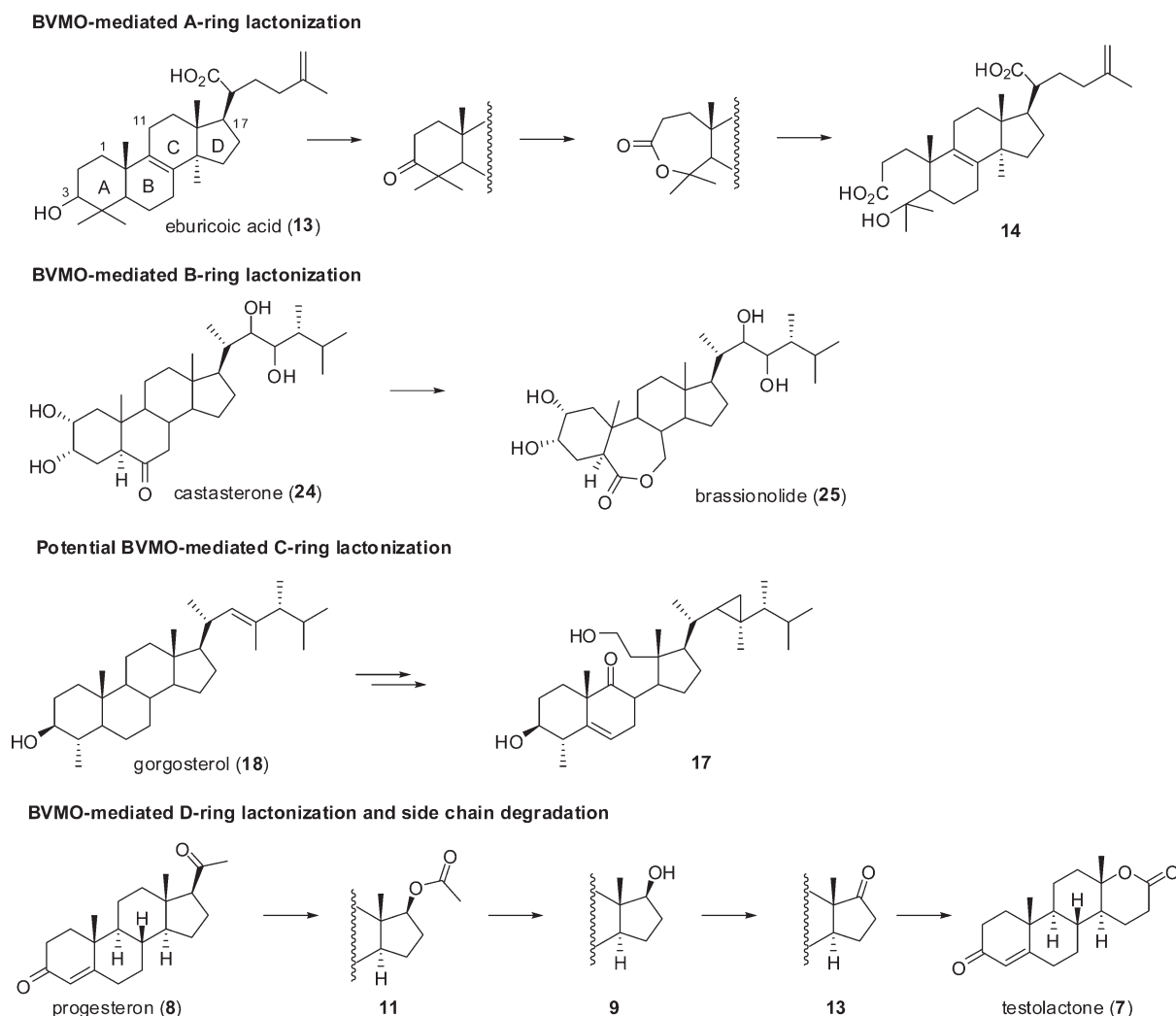


Figure 6. BVMO-mediated oxidations of steroids.

In 1963 the first logical link between the D-ring lactonization of steroids, the biochemical oxidation of camphor by a bacterium (1959), and the chemical Baeyer–Villiger oxidation was made by Prairie and Talalay.⁸¹ They partially purified and identified two steroid-induced enzymes, a dehydrogenase and a “lactonizing” enzyme system, and could show that the enzyme uses molecular oxygen for the lactonization by performing tracer studies with oxygen.⁸¹ Further mechanistic studies on the oxidative cleavage of the side chain of C₂₁ steroids with *C. radicola* (ATCC 11011) by Rahim and Sih led to the identification of NADPH as an essential cofactor for the oxygenase derived from this fungal species.⁸² While the first substrate acceptance studies and screenings for new fungal strains appeared in the literature,⁸³ Fried and co-workers investigated the transformation of the fungal metabolite eburicoic acid (13) to biologically active steroids.⁸⁴ *Glomerella fusarioides* ATCC 9552 was found to convert eburicoic acid into compound 14, and the authors suggested a plausible mechanism for the oxidative A-ring degradation and noted the similarity of those isolated by Turfitt 16 years earlier.⁸⁴ Two other BVMO-mediated A-ring cleavages of steroids have been reported, namely, the oxidation of tomatidone (15) by *Gymnoascus reesii* in 1983⁸⁵ and the A-ring degradation of 17 β -acetoxy-5 α -androstane-3-one (16) and

testosterone (9) by the thermophilic fungus *Myceliophthora thermophila* CBS 117.65.⁸⁶

The preparation of 9(11)-secosteroid (17) with crude enzyme extracts of the marine gorgonian *Pseudopterogorgia americana* from gorgosterol (18), cholesterol (19), stigmasterol (20), and progesterone (8) was reported by Kerr and co-workers (Figure 6).⁸⁷ Although neither of the enzymes was ever purified or characterized nor were other studies published, it can be assumed that BVMOs are involved in the oxidative C-ring cleavage of these steroids.

In 1986, 10 years after the first purification of a bacterial monooxygenase by Trudgill and co-worker, Itagaki succeeded in the purification of a steroid monooxygenase (SMO) from *C. radicola* ATCC 11011 by affinity chromatography on a pregnenolone–Sepharose column. NADPH was identified as essential electron donor and the enzyme displayed substrate specificity toward a wide range of C₂₁-20-ketosteroids.^{42,88} Further studies revealed that the purified enzyme is able to catalyze the oxidation of progesterone (8) to testosterone acetate (11) as well as the lactonization of androstendione (13).⁸⁸ The same group reported on the first purification and characterization of a bacterial steroid MO,⁶¹ which was later sequenced and overexpressed as recombinant protein in *E. coli*.⁶² Comparison with the previously

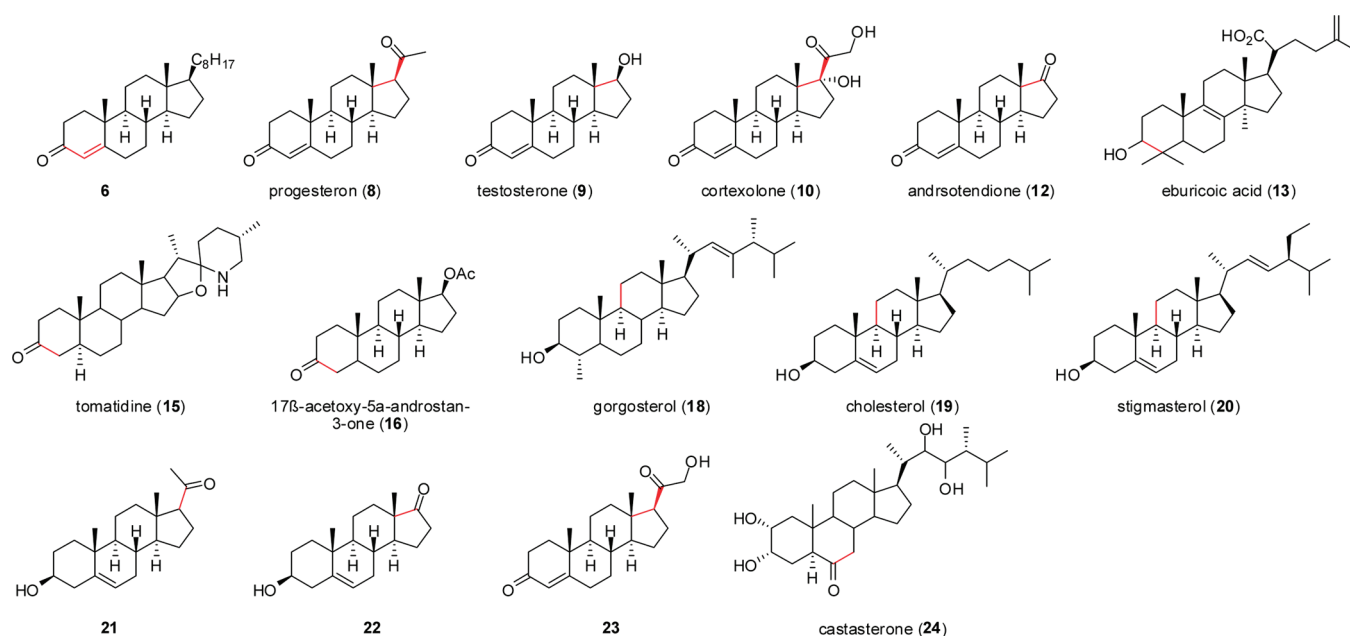


Figure 7. Selected steroidal substrates for BVMO-mediated oxidations. Sites for the oxidative cleavage are indicated by red bonds.

isolated fungal MO from *C. radicola* ATCC 11011 revealed a much narrower substrate acceptance for only progesterone-type steroids, while C_{17} -ketosteroids were not transformed.^{61,62}

Research efforts in chemoenzymatic production of pharmaceutically valuable steroids and sterols are still ongoing and focus either on the optimization of existing processes (mainly regio- and stereoselective hydroxylations at C-11) or on identification of potentially useful and profitable bioconversions.⁸⁹ Therefore it is not surprising that several fungal strains have been reported to exhibit BVMO activity on steroids. Notable reports in the last 10 years are the oxidative side chain degradation of 3β -hydroxy-pregn-5-en-20-one (21) by *Exophiala jeanselmei* var. *lecanii-corni*,⁹⁰ testolactone production from various testoids by *Penicillium notatum* (KCH 904)⁹¹ and *Penicillium citreo-viride* (ACCC 0402),⁹² D-ring lactonization of 3-oxosteroids by *Trichoderma hamatum* (KCh25),⁹³ whole cell mediated BV oxidation of testosterone (9) by *Rhizopus stolonifer* (ATCC 10404),⁹⁴ the oxidation of both steroidal 4-en-3-ketones derivatives (e.g., 12, Figure 7) and 5-en- 3β -alcohols (e.g., 22, Figure 7) by *Penicillium camemberti* AM83,⁹⁵ and A-ring degradation of 17β -acetoxy- 5α -androstan-3-one (16) and testosterone (9) by the thermophilic fungus *Myceliophthora thermophila* CBS 117.65.⁸⁶ In addition to the continuing quest for new fungal strains with unique biocatalytic abilities, strains such as *Penicillium lilacinum*^{79d,80b–80e,81} and *Aspergillus tamarii*,^{78b} which have been shown previously to possess BVMO activity, were revisited and applied as whole cell biocatalysts.⁹⁶ The group of Hunter investigated the metabolic pathways within *A. tamarii* with several steroids.^{96b–f} Steroidal substrates with steroidal side chains (e.g., 23, Figure 7) gave D-ring lactones,^{96b} whereas steroidal probes with switched functionalities, alcohol, acetate, or ketone functionality at C-3 and enone functionality at the D-ring, gave exclusively hydroxylations.^{96c} In contrast to all recent whole cell mediated fungal transformations, the group of Ottolina studied the enzymatic action of a cloned cyclopentadecanone monooxygenase (CPDMO) on different steroids. The bioconversions were conducted either with purified enzymes

with *in situ* cofactor regeneration or with whole cells overexpressing the recombinant mutant S216A of CPDMO. Among the 33 tested steroids, 12 (3-keto or 17-ketosteroids) were shown to be substrates for CPDMO, and both A- and D-ring steroidal lactones were isolated.⁹⁷

In addition to the numerous reports on BV oxidations of steroids, a few examples of microbial BV oxidations of the structurally closely related triterpenes have been described.⁹⁸

4.1.2. Plant Cell Mediated Baeyer–Villiger Oxidations of Steroids. The first evidence that plant cells are able to catalyze an enzyme-mediated BV reaction was reported by the group of Fujioka while investigating the biosynthesis of brassinosteroids (a class of plant steroids responsible for plant growth and development).⁹⁹ Feeding experiments using labeled castasterone (24) showed that different cell lines of *Catharanthus roseus* could oxidize the B-ring of the plant steroid to give brassinolide (25) (Figure 6).¹⁰⁰ Likewise, Schneider and co-workers reported an enzyme-mediated BV reaction in the biosynthesis of brassinosteroids in tomato cells.¹⁰¹ Unlike the enzymes derived from bacterial or fungal sources, the B-ring lactonizations of plant steroids are catalyzed by members of the CYP85A family of cytochrome P450 monooxygenases.¹⁰² A *Pichia pastoris* clone coexpressing the previously identified CYP85A2 from *Arabidopsis* and P450 reductase was shown to oxidize not only castasterone (24) but two other related steroids as well.¹⁰³

4.2. Baeyer–Villiger Monooxygenases in the Metabolism of Terpenoids

In 1959, more than 10 years after the first indications of the existence of an enzymatic version of the Baeyer–Villiger oxidation during the bioconversion of steroids, Bradshaw et al. reported the microbial degradation of (+)-camphor (26) with *Pseudomonas putida* isolated from sewage sludge.¹⁰⁴ Although no biochemical studies were presented in this initial paper, the isolation of two lactone metabolites indicated the existence of BVMO activity in *P. putida*. Similar oxidative activity was found

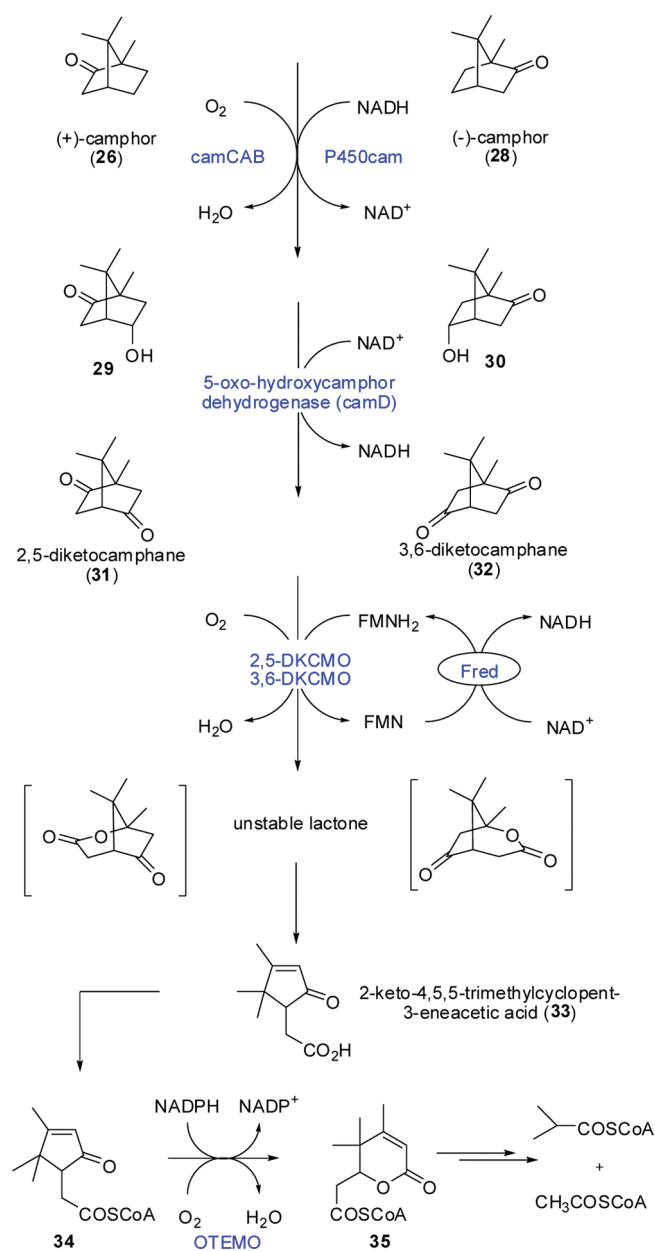


Figure 8. Degradation of (+)- and (-)-camphor by *Pseudomonas putida* ATCC 17453. Fred is a flavin reductase.

in a *Corynebacterium* sp., which was capable of converting racemic fenchone (27) into 1,2- and 2,3-fencholides when grown on (+)- or (-)-camphor (26, 28) as carbon source.¹⁰⁵

The complete elucidation of the enzymology of the enzyme-mediated degradation of (+)- and (-)-camphor (26, 28) engaged the research groups of Gunsalus and Trudgill for more than 30 years and is shown in Figure 8.¹⁰⁶ The first step in the metabolism of camphor by *Pseudomonas putida* (ATCC 17453 or NCIMB 10007) is the hydroxylation of either isomer by a cytochrome P450-containing enzyme complex, followed by the action of two discrete dehydrogenases, each with a preference for the isomer used for growth.¹⁰⁶ⁱ Oxidation of the two enantiomeric diketocamphor derivatives (2,5-diketocamphane (31) from (+)-camphor and 3,6-diketocamphane (32) from (-)-camphor) is mediated by two enantiocomplementary isozymic protein

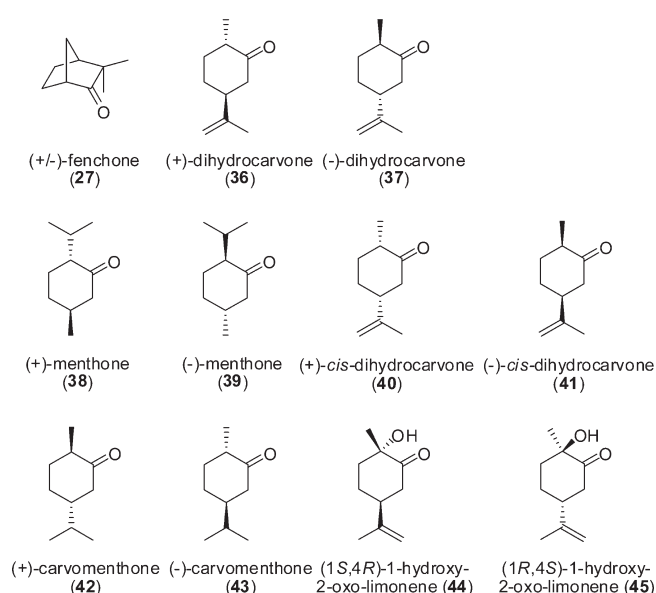


Figure 9. Terpenes identified as substrates for BVMOs.

complexes, which were named after their natural substrates, 2,5-DKCMO and 3,6-DKCMO (a mixture of both is referred to as MO1).^{70,106e} Each MO consists of a loose complex of a NADH dehydrogenase and a FMN-containing oxygenation component making both protein complexes type 2 BVMOs. All involved proteins were purified to homogeneity and biochemically characterized. In contrast to the hydroxylase complex and the two dehydrogenases, both BVMOs are absolutely specific for substrates of either enantiomeric series of camphor-ketones.¹⁰⁶ⁱ The produced lactones undergo spontaneous elimination to give cyclopentylacetic acid derivative 33, which serves as a substrate for an inducible coenzyme A ester synthetase. The activated thioester 34 is then oxidized by a third BVMO, namely, 2-oxo- Δ^3 -4,5,5-trimethylcyclopentenylacetic acid MO (OTEMO or previously called MO2), to give compound 35, which is further metabolized. OTEMO was purified to homogeneity and was found to consist of two identical subunits.⁵⁵ The oxygenase was specific for NADPH as an electron donor and contained stoichiometric amounts of FAD as a prosthetic group, classifying OTEMO as a type 1 BVMO in contrast to 2,5-DKCMO and 3,6-DKCMO. Crystallization and preliminary X-ray diffraction studies of the oxygenating subunit of 2,5- and 3,6-DKCMO from *P. putida* (NCIMB 10007) were conducted by the group of Littlechild.¹⁰⁷

Interestingly a different metabolic degradation of (+)-camphor (26) was observed with *Mycobacterium rhodochrous*. The pathway, formation of 2,6-diketocamphor followed by β -diketohydrolase-mediated ring cleavage to yield a cyclopentylacetic acid, which is further degraded by the action of a BVMO similar to the action of OTEMO, was deduced from the identification of intermediates and their use in enzymatic studies.¹⁰⁸ Croteau and co-workers showed that the metabolism of (+)-camphor is not restricted to microorganisms.¹⁰⁹ They found evidence that (+)-camphor (26) was transformed to 1,2-campholide in mature leaves of flowering sage plants similar to the action of 2,5-DKCMO.

In addition, Shukla and co-workers isolated a *Rhodococcus* sp. from sewage, which was able to grow on (-)-menthol (39) as a sole source of carbon.¹¹⁰ The authors proposed that one step in the metabolic pathway of menthol involved an enzymatic BV

oxidation. Monocyclic monoterpene ketone monooxygenase (MMKMO), a BVMO from *Rhodococcus erythropolis* DCL14 was purified and characterized by Werf.⁵⁹ The protein was shown to be monomeric with a molecular mass of 60 kDa. The NADPH-dependent and FAD-containing type 1 BVMO was shown to be involved in the metabolism of the three naturally occurring monoterpenes, menthol, carveol, and limonene.¹¹¹ Substrate specificity studies showed that MMKMO oxidizes a wide range of terpenones as well as cycloalkanones.⁵⁹ The oxidation of terpenones has also been reported with crude enzyme extracts of *Corynebacterium* sp. and whole cells of *Trichosporum cutaneum* CCT 1903.¹¹²

Preparative scale biotransformations of terpenones are rather scarce. Initial studies were conducted with immobilized enzyme preparations of CHMO from *Acinetobacter* NCIMB 9871 by Whiteside and co-workers in 1989.¹¹³ Lactones were prepared on a 30–80 mmol scale from (+)- and (–)-fenchone (27), (+)-camphor (26), and (+)-dihydrocarvone (40). NADPH was regenerated *in situ* with glucose-6-phosphate and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*.¹¹³ Three years later menthone and dihydrocarvone were subjected to whole cell fermentations with either *Acinetobacter* NCIMB 9871 or *Acinetobacter* TD63 by Alphand and Furstoss.¹¹⁴ (+)-Menthone (38, Figure 9) proved to be an excellent substrate for both native strains, in contrast to the (–)-isomer (39), which was not converted at all. Biotransformations with dihydrocarvone, 40 and 41, yielded two regioisomeric lactones in excellent yield and optical purity. The formation of enantiocomplementary regioisomeric lactones from terpenones by several recombinant whole cell expression systems of BVMOs was investigated in more detail by the group of Mihovilovic and will be discussed in section 4.5.5 of this review.^{68,115}

4.3. Degradation of Linear, Cyclic, and Aromatic Ketones

This section of the review will cover BVMO-mediated oxidations of linear, cyclic, and aromatic ketones to nonchiral lactones and esters. Since the preparation of simple lactones, such as ϵ -caprolactone, can be easily achieved by peracetic acid mediated BV oxidations, the synthetic value of enzyme-catalyzed oxidations in this context is questionable except in the context of environmental reasons. Nevertheless, studies on the BVMO-catalyzed oxidation of simple ketones were vital for the development of modern BVMO-based technologies and asymmetric transformations, which will be covered in following sections. Moreover isolation of microbes that can utilize simple ketones as a sole source of carbon must be considered as the most successful strategy to identify new BVMOs until the appearance of genome mining. Application of this strategy led to isolation and identification of wild-type cells bearing some of the best studied BVMOs to date.

4.3.1. Linear Ketones. As early as 1923, Supniewski reported that *Bacillus pyocyaneus* would accumulate acetic acid and formic acid when grown in a medium containing 0.23% acetone. However this report cannot be considered as proof of the involvement of an enzymatic BV oxidation in the degradation of acetone.¹¹⁶ Another early report indicating an oxidative carbon–carbon cleavage of acetone was published by Goepfert in 1941, who detected formaldehyde production during the growth of *Fusarium* on isopropanol, acetone, and 1,2-propanediol.¹¹⁷ More than 40 years after the report of Supniewski, the degradation of propane (46) with *Mycobacterium* JOB-5 was suggested to proceed via isopropanol and acetone to acetol (47), followed by a carbon–carbon cleavage to yield acetate and a C₁ unit.¹¹⁸ The presence of an enzyme in

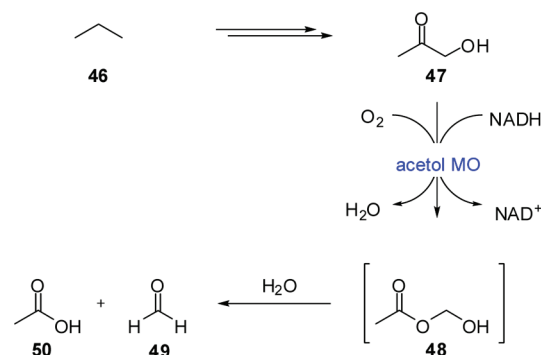


Figure 10. Acetol oxidation in propane metabolism.

Mycobacterium Py1 capable of transforming acetol (47) into acetate and formaldehyde presumably via hydroxymethylacetate (48) as intermediate was established by Hartmans and de Bont (Figure 10).¹¹⁹ A BVMO that converts acetone into methyl acetate was described by Sakai and co-workers in 2007.^{44,120} This BVMO-encoding gene was identified in *Gordonia* sp. strain TY-5 and cloned in *E. coli*. Acetone monooxygenase (ACMO) was purified to near homogeneity in four steps and showed high activity toward C₃ to C₁₀ linear methyl ketones as well as C₄ to C₆ cyclic ketones.¹²⁰

The first report on the characterization of oxidative intermediates from the metabolism of methyl ketones other than acetone was provided by Forney, Markovetz, and Kallio in 1967.¹²¹ 2-Tridecanone was converted by either *Pseudomonas multivorans* or *Pseudomonas aeruginosa* into undecyl acetate or the corresponding hydrolyzed fragments of the ester.¹²¹ The same group established that crude enzyme extracts of *P. aeruginosa* converted the methyl ketone derivative in the presence of oxygen and NADH or NADPH.¹²² Subsequent studies with ¹⁸O₂ confirmed the incorporation of molecular oxygen in the methyl ketone derived ester and these experiments provided a proof for an enzymatic BV oxidation.¹²³ Similar enzymatic activity was found in *Pseudomonas cepacia* and further studies allowed for the purification of a methyl ketone oxidizing BVMO. Enzymatic assays showed that esters are formed from C₇ to C₁₄ methyl ketones and valerolactone from cyclopentanone.⁵⁶

Similar mechanisms for the oxidative degradation of tetradecane and 1-tetradecene by a *Penicillium* sp.,¹²⁴ as well as 2-butanone by *Nocardia* strain LSU-169,¹²⁵ were described. Studies of the methyl ketone degradation pathway in *Pseudomonas veronii* MEK700 by Engesser and co-workers revealed the involvement of a BVMO.⁵⁸ The BVMO-encoding gene Meka, which was identified by transposon mutagenesis, was cloned and expressed in *E. coli*. Initial substrate studies with either purified enzyme preparations or recombinant whole cells showed activity toward aliphatic, aromatic, and cyclic ketones.⁵⁷ In addition BVMOs from *Pseudomonas fluorescens* DSM 50106⁵¹ and from *Pseudomonas putida* KT2440⁵⁴ were cloned and functionally expressed in *E. coli* by Bornscheuer and co-workers. Both MOs showed high specificity toward the degradation of aliphatic open-chain ketones.

4.3.2. Alicyclic Ketones. Examples for alicyclic compound degrading microbes are the cyclohexanol metabolizing strains *Nocardia globerula* CL1¹²⁶ and *Acinetobacter* NCIMB 9871¹²⁷ cyclohexanone degrading strain *Rhodococcus* sp. strain HI-31,⁶³ cyclohexane degrading strain *Xanthobacter* sp.,¹²⁸ cyclopentanol degrading strain *Pseudomonas* sp. NCIMB 9872,²¹ cyclohexan-1,2-diol degrading

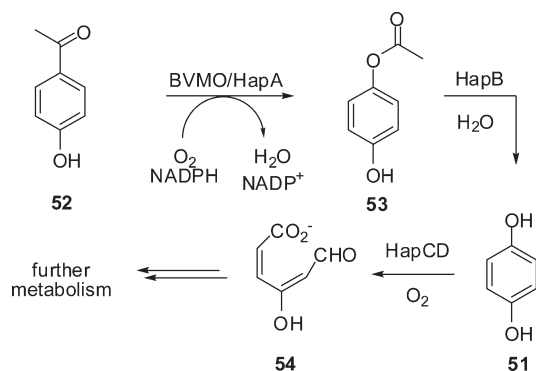


Figure 11. Proposed metabolic pathway of 4-hydroxyacetophenone by *P. fluorescens* ACB.

strain *Acinetobacter* TD63,¹²⁹ the cyclododecanone metabolizing strain *Rhodococcus ruber* CD4 DSM 44394,¹³⁰ cyclopentadecanone degrading strain *Pseudomonas* sp. strain HI-70,^{23b} and the cyclic ketone and alcohol degrading strain *Brevibacterium epidermidis* HCU.^{39b} In each case, the metabolism of alicyclic compounds follows a similar pathway, in which an enzymatic BV oxidation plays a key role as described in section 3.2 of this review (Figure 3). In two consecutive publications, Trudgill and co-workers described the purification of three different BVMOs, two CHMOs from *Acinetobacter* NCIMB 9871 and *Nocardia globerula* CL1¹³ and CPMO from *Pseudomonas* NCIMB 9872.¹⁴ The common feature of the three BVMOs were their dependency on FAD and NADPH as cofactors, and in addition the ratio of FAD to enzyme was found to be 1:1.

In 1988, the first BVMO-encoding gene, CHMO gene from *Acinetobacter* NCIMB 9871, was isolated by immunological screening methods by Walsh and co-workers.³² The cloning and sequencing allowed for the prediction of the amino acid sequence, although some 13 years after the initial sequence was certified incorrect by resequencing of the CHMO-encoding gene (GenBank accession number AB006902)^{21b} and by mass spectroscopy analysis of the protein.⁷³ Nevertheless, the initial cloning facilitated heterologous expression in yeast by the groups of Kayser and Stewart^{33a} and subsequent overexpression in *E. coli* by Woodley and co-workers making use of an arabinose-inducible promoter in the pBAD expression system.³⁴ Subsequently, the CPMO-encoding gene from the prototypical *Comamonas* (previously *Pseudomonas*) sp. NCIMB 9872 was also cloned, sequenced, and overexpressed in *E. coli*.¹⁸ The CPMO sequence was established to be 37% identical to that of CHMO. Other noteworthy examples are the use of mRNA differential display, which led to the identification and cloning of two CHMO encoding genes in *Brevibacterium* sp. strain HCU by Rouviere and co-workers^{39a} or the use of highly degenerate primers based on the sequences of known and putative BVMOs, which allowed for functional expression of BVMOs from *Comamonas*, *Xanthobacter*, and a *Rhodococcus* strain.⁴¹ A ligation independent cloning strategy was used by Grogan and co-workers to evaluate the 23 putative BVMO genes from the *Rhodococcus jostii* RHA1 genome, which led to the functional expression of 13 BVMOs.⁶⁰ A complete list of recombinant BVMOs is presented in Table 2, section 3.3.

4.3.3. Acetophenone and Aromatic Ketones. The metabolism of aromatic ketones was first reported for *Arthrobacter* sp. 2 by Cripps in 1975. The organism was found to grow on acetophenone as a sole carbon source, and cell-free extracts were shown to convert

acetophenone to phenol in a NADPH and oxygen consuming reaction.¹³¹ A degradative pathway of acetophenone was suggested by the author as follows: formation of phenylacetate by the action of a MO, followed by hydrolysis and oxidation of the phenol to catechol, which is further metabolized by *Arthrobacter* sp. 2 by the β -oxoadipate pathway.¹³¹ A similar metabolic pathway was described for the oxidative degradation of racemic 1-phenylethanol by *Arthrobacter* sp.¹³² The production of hydroquinone (51) from 4-hydroxyacetophenone (52) with crude enzyme extracts of *Pseudomonas putida* JD1 supported the previously proposed catabolic pathway for the degradation of acetophenone by *Arthrobacter* and the involvement of an enzymatic BV reaction, since alternative pathways would lead to isolation of 4-hydroxy benzoic acid, which was not detected.¹³³ The oxidative cleavage of an anthraquinone derivative by *Aspergillus terreus* was reported by Sankawa and co-workers, and it was shown that two enzymes were required to carry out the oxidative reaction with an absolute requirement for NADPH.¹³⁴ BVMO activity toward chlorinated acetophenones was found in *Alcaligenes* sp. strain ACA and *Pseudomonas fluorescens* ACB. Chlorinated phenylacetates could be isolated when biotransformations were performed in the presence of an esterase inhibitor. NADPH-dependent MO activity in crude enzyme extracts was strongly inhibited by *meta*- or *ortho*-substituted phenols.¹³⁵ Detailed analysis of intermediates in the degradation of fluorene by *Arthrobacter* sp. strain 101 indicated three metabolic pathways, two of them involving enzymatic BV reactions.¹³⁶ The first aromatic ketone degrading enzyme to be purified was the aryl ketone MO from *P. putida* JD1,⁵² a strain which was previously shown to be responsible for the conversion of aromatic ketones.¹³³ Biochemical characterization showed that the enzyme contains one molecule of FAD and has a molecular weight of $M_r \approx 70$ kDa. Comparison of the protein sequence of the aryl ketone MO with CHMO from *Acinetobacter* sp. revealed high similarity in an internal part of the protein.⁵² Purification, gene cloning, sequence analysis, and biochemical characterization of 4-hydroxyacetophenone monooxygenase (HAPMO) from *P. fluorescens* ACB was reported by the group of Janssen one year after purification of the aryl ketone MO.⁴⁹ Purified HAPMO was reported to be a FAD-containing homodimer of 140 kDa and significant similarities ($\sim 33\%$) in the protein sequence were found with CHMO and SMO. Initial substrate screening revealed that HAPMO oxidizes a wide range of substituted acetophenones to the corresponding phenylacetates. In addition a detailed study on the complete catabolic pathway of 4-hydroxyacetophenone (52) by *P. fluorescens* ACB was published (Figure 11).¹³⁷

The first application of genome mining, using the 2002 identified BVMO-specific sequence motif,¹³⁸ allowed for the identification of a gene encoding a BVMO in the moderately thermophilic actinomycete *Thermobifida fusca*.⁶⁶ Biochemical characterization of the recombinant protein classified the enzyme as a type 1 BVMO. Substrate screening revealed a good substrate acceptance for aromatic and aliphatic ketones, with phenylacetone being the best substrate, and therefore the BVMO from *Thermobifida fusca* was named phenylacetone MO (PAMO). Suffice it to say that the first structural elucidation of a BVMO is that of PAMO, which will be discussed in more detail in a following section.⁶⁵

Screening of fungal strains for acetophenone MO activity was conducted by the group of Andrade and the authors identified *Emericella nidulans* CCT 3119 as potential biocatalyst.¹³⁹ In 2009, a pseudomonad acetophenone MO encoding gene from *Pseudomonas putida* JD1 was cloned and functionally expressed in *E. coli* by the group of Bornscheuer.⁵³

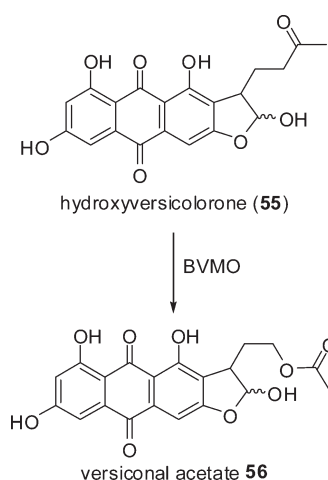


Figure 12. Conversion of hydroxyversicolorone (55) to versiconal acetate 56 in aflatoxin biosynthesis.

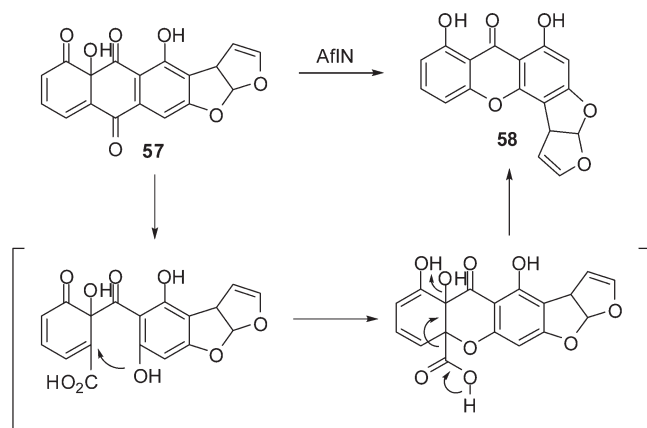


Figure 13. Anthraquinone to xanthone transformation in fungal biosynthesis.

4.4. Baeyer–Villiger Monooxygenases in Biosynthesis and Prodrug Activation

In addition to the widespread occurrence of BVMOs in the degradation of steroids, terpenoids, alicyclic, and aromatic ketones, numerous BVMOs were identified in the biosynthesis of polyketides, mycotoxins, and antibiotics. Extensive studies on the aflatoxin and related polyketide biosyntheses mainly by the group of Townsend revealed the involvement of two BV reactions. The first oxidation, conversion of hydroxyversicolorone (55) to versiconal acetate 56 occurs relatively early in the assembly of this potent class of mycotoxins and was established by ^{18}O -incorporation experiments (Figure 12).¹⁴⁰ The second BV oxidation was found to be involved in the conversion of the anthraquinone derivative 57 to compound 58 by a cytochrome P450 MO and a short-chain NADPH reductase as shown in Figure 13.¹⁴¹ C–C bond cleavage adjacent to a ketone functionality and therefore very likely being a BV oxidation was observed in the biosynthesis of antitumor antibiotics vineomycins A1 and B1 by *Streptomyces mateusis* subsp. *vineus*.¹⁴² It was proposed that the pyrone ring of simaomicin α^1 , a hexacyclic xanthone antibiotic is formed by a BV oxidation of a carbocyclic intermediate followed by decarboxylation and ring closure.¹⁴³ A similar transformation in the biosyntheses of xanthone antibiotics was

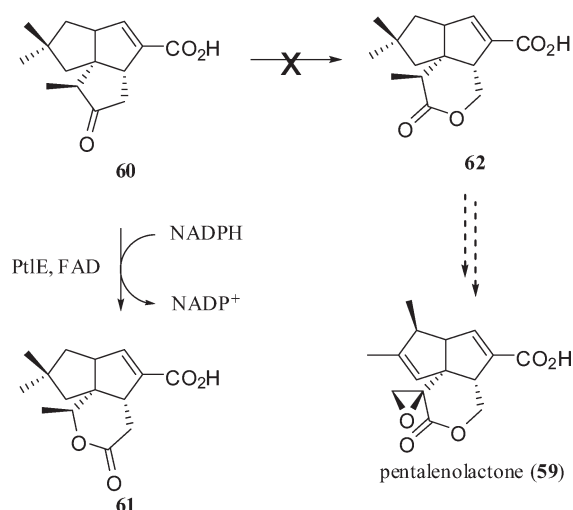
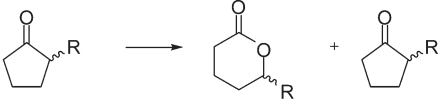


Figure 14. PtlE-mediated oxidation of 1-deoxy-11-oxopentalenic acid (60).

observed by the group of Rohr, who proposed that the xanthone framework of lysolipins is derived upon BV oxidation of a quinone intermediate.¹⁴⁴ A BV oxidation in the biosynthesis of the polyketide DTX-4, a precursor of the diarrhetic shellfish poison okadaic acid, was described by the group of Wright.¹⁴⁵ In addition, the authors hypothesized that BV oxidations are responsible for the elimination of single carbon units of polyketide chains by formation of a cyclopropanone unit from a α -diketide followed peroxide attack and Favorski-type rearrangement. The seven-membered lactone unit in urdamycin L, a shunt product obtained in trace amounts during biosynthesis studies of urdamycin A with a mutant strain of *Streptomyces fradiae* TŪ 2717 was attributed to the action of a BVMO.¹⁴⁶ BVMO-mediated oxidations were also reported to play a significant role in the transformation of polyketide-derived angucyclinones to the tetracyclic cores of the anticancer antibiotics gilvocarcins and jadomycins.¹⁴⁷ The involvement of a BV oxidation in the biosynthesis of chlorothricin by *Streptomyces antibioticus* DSM 40725 was confirmed by Liu and co-workers during identification of the biosynthetic gene cluster.¹⁴⁸ Mithramycin (5), a member of the aureolic acid group of antitumor drugs, is produced by *Streptomyces argillaceus* via multiple cyclization of a decaketide chain to a tetracyclic intermediate, which is further methylated and glycosylated to give premithramycin B (3). The fourth ring of this intermediate is oxidized by the action of a BVMO, and further steps lead to the formation of mithramycin.¹⁴⁹ Further studies by the group of Rohr led to the overexpression and biochemical characterization of MtmOIV, the protein responsible for the BV reaction in the biosynthetic pathway of mithramycin.²⁰ Interestingly MtmOIV is very distinct from bacterial type 1 and type 2 BVMOs and is considered as an atypical BVMO. Moreover the structure of MtmOIV was solved by X-ray crystallography and will be discussed in more detail in a following section.⁷¹ PtlE, a type 1 BVMO from *Streptomyces avermitilis*, was discovered by genome mining during studies on the biosynthesis of pentalenolactone (59). Heterologously expressed PtlE converted 1-deoxy-11-oxopentalenic acid (60) to neopentalenolactone D (61) and not the expected pentalenolactone D (62) a precursor of pentalenolactone (Figure 14).²⁷ Therefore the action of PtlE allows access to a whole new class of



Substrate	R	Biocatalyst ^a	<i>E</i> – value or % <i>ee</i> ^b	Reference
63a	Me	CHMO _{Acineto} - yeast	3.6 (n. r.)	160c
		CPDMO - <i>E. coli</i>	15	23b
63b	Et	CHMO _{Acineto} - yeast	3.7 (n. r.)	160c
63c	<i>n</i> -Pr	CHMO _{Acineto} - yeast	30 (-)	160c
63d	<i>iso</i> -Pr	CHMO _{Acineto} - yeast	(-)	160b
63e	allyl	CHMO _{Acineto} - yeast	2.3 (-)	160c
		CHMO _{Acineto} - <i>E. coli</i>	2.3 (<i>R</i>)	161
		CPMO - <i>E. coli</i>	0	161
63f	<i>n</i> -Bu	MO1 - <i>P. putida</i> NCIMB 10007	5 (-)	156
		MO2 - <i>P. putida</i> NCIMB 10007	104 (-)	156
		CHMO _{Acineto} - yeast	>200 (-)	160c
		CHMO _{Acineto} - <i>E. coli</i>	200 (<i>S</i>)	161
		CPMO _{Coma} - <i>E. coli</i>	1.5 (<i>S</i>)	161
63g	<i>iso</i> -Bu	CHMO _{Acineto} - yeast	16	161
63h	<i>n</i> -Pent	<i>Acinetobacter</i> NCIMB 9871	<i>ee</i> _P = rac	155
64i	<i>cyclo</i> -Pent	CHMO _{Acineto} - <i>E. coli</i>	1.3	161
		CPMO - <i>E. coli</i>	1.6	161
63j	<i>n</i> -Hex	MO2 - <i>P. putida</i> NCIMB 10007	52 (-)	156
		CHMO _{Acineto} - yeast	>200 (-)	160c
63k	<i>n</i> -Hept	<i>Ps. oleovorans</i>	not reported	151
		<i>Acinetobacter</i> NCIMB 9871	<i>ee</i> _P = 95 (<i>S</i>)	155
63l	<i>n</i> -Oct	MO1 - <i>P. putida</i> NCIMB 10007	23 (-)	156
		MO2 - <i>P. putida</i> NCIMB 10007	63 (-)	156
		CHMO _{Acineto} - yeast	>200 (-)	160c
63m	<i>n</i> -Non	<i>Acinetobacter</i> NCIMB 9871	<i>ee</i> _P = 85 (<i>S</i>)	155
63n	<i>n</i> -Dec	MO1 - <i>P. putida</i> NCIMB 10007	20 (-)	156
		MO2 - <i>P. putida</i> NCIMB 10007	7 (-)	156
63o	<i>n</i> -Undec	<i>Acinetobacter</i> NCIMB 9871	<i>ee</i> _S = 75 (<i>R</i>) <i>ee</i> _P = 45 (<i>S</i>)	154
		CHMO _{Acineto} - yeast	>200 (-)	160c
63p	Bn	CHMO _{Acineto} - <i>E. coli</i>	7.3	161
		CPMO - <i>E. coli</i>	1.3	161
63q	CH ₂ CO ₂ Et	MO2 - <i>P. putida</i> NCIMB 10007	>100 (<i>R</i>)	157a
		CPMO - <i>E. coli</i>	8	161
63r	CH ₂ CH ₂ OAc	MO2 - <i>P. putida</i> NCIMB 10007	>100 (<i>R</i>)	157a
		CPMO - <i>E. coli</i>	4.6 (<i>R</i>)	161
63s	CH ₂ OTBS	MO2 - <i>P. putida</i> NCIMB 10007	3 (<i>R</i>)	157a
63t	CH ₂ OCH ₂ O(CH ₂) ₂ OC H ₃	MO2 - <i>P. putida</i> NCIMB 10007	58 (<i>R</i>)	157a
63u	CH ₂ CH ₂ CH ₂ Br	CHMO _{Acineto} - yeast	128	161
		CPMO - <i>E. coli</i>	5.7	161
63v	CH ₂ OH	CHMO _{Acineto} - yeast	2.3 (<i>R</i>)	161
		CPMO - <i>E. coli</i>	2.8 (<i>R</i>)	161
63w	CH ₂ OMe	CHMO _{Acineto} - <i>E. coli</i>	2.8	161
		CPMO - <i>E. coli</i>	3.7	161
63x	CH ₂ Oallyl	CHMO _{Acineto} - <i>E. coli</i>	69 (<i>R</i>)	161
		CPMO - <i>E. coli</i>	13 (<i>R</i>)	161

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation or absolute configuration of product is given in parentheses.

Figure 15. Kinetic resolution of 2-substituted cyclopentanones.

pentalenolactone derivatives. It is noteworthy that PtIE from *Streptomyces avermitilis* and MtmOIV from *Streptomyces argillaceus* are among the few BVMOs that have been characterized with their true natural substrates.^{26,27}

Four years after the identification of a protein (EtaA) from *Mycobacterium tuberculosis*,¹⁵⁰ which is responsible for the conversion of ethionamide into an active antitubercular drug, it was shown by Fraaije and co-workers that the enzyme is a type 1

BVMO.⁴⁶ This enzyme, represented by Rv3854c, clusters with two other mycobacterial BVMO sequences (Figure 5), as well as the more recently described *P. putida* KT 2440 BVMO.⁵⁴ Like the multiple BVMO sequences from *Rhodococcus jostii* RHA1, all six genomic sequences in *M. tuberculosis* H37Rv were cloned by a “ligation free” strategy and expressed in *E. coli*. Substrate screening revealed that four clones were active; however the prodrug ethionamide was not tested as substrate.⁴⁵

65a-o 66a-o 65a-o

Substrate	R	Biocatalyst ^a	<i>E</i> - value or % ee (yield) ^b	Reference
65a	Me	MO2 - <i>P. putida</i> NCIMB 10007	4 (S)	159
		<i>Acinetobacter</i> TD63	6 (S)	159
		CHMO _{Acineto} - yeast	10 (S)	160a
		CDMO - <i>E. coli</i>	>200 (S)	162
		CPDMO - <i>E. coli</i>	>200 (S)	23b
65b	¹³ CH ₃ / CD ₃	CHMO _{Acineto} - purified	not reported	152
65c	Et	MO2 - <i>P. putida</i> NCIMB 10007	2 (S)	159
		<i>Acinetobacter</i> TD63	58 (S)	159
		CHMO _{Acineto} - yeast	>200 (S)	160a
65d	<i>n</i> -Pr	CHMO _{Acineto} - yeast	>200 (S)	160a
65e	<i>iso</i> -Pr	CHMO _{Acineto} - yeast	>200 (R)	160a
65f	allyl	CHMO _{Acineto} - yeast	>200 (R)	160a
		CHMO _{Xantho} - <i>E. coli</i>	99 (R)	68
65g	<i>n</i> -Bu	MO2 - <i>P. putida</i> NCIMB 10007	30 (S)	159
		<i>Acinetobacter</i> TD63	>100	159
		CHMO _{Acineto} - yeast	>200 (S)	160a
65h	<i>n</i> -Hex	MO2 - <i>P. putida</i> NCIMB 10007	12 (S)	157a
		<i>Acinetobacter</i> TD63	>100 (S)	159
65i	<i>n</i> -Oct	MO2 - <i>P. putida</i> NCIMB 10007	14 (S)	157a
65j	<i>n</i> -Non	MO2 - <i>P. putida</i> NCIMB 10007	5 (S)	159
		<i>Acinetobacter</i> TD63	20 (S)	159
65k	Ph	MO2 - <i>P. putida</i> NCIMB 10007	60 (R)	159
		<i>Acinetobacter</i> TD63	>100 (R)	159
		CHMO _{Xantho} - <i>E. coli</i>	>200 (R)	68
65l	Bn	MO2 - <i>P. putida</i> NCIMB 10007	4 (R)	159
		<i>Acinetobacter</i> TD63	>100 (R)	159
		CHMO _{Xantho} - <i>E. coli</i>	>200 (R)	68
65m	CH ₂ CO ₂ Et	MO2 - <i>P. putida</i> NCIMB 10007	63 (R)	157a
		CHMO _{Acineto} - purified	ee _S = 64 (S) (60%) ee _P = 99 (R) (39%)	198
65n	CH ₂ CH ₂ OAc	MO2 - <i>P. putida</i> NCIMB 10007	17 (R)	157a
		CPMO - purified	5 (S)	157a
		CHMO _{Acineto} - purified	ee _S = 76 (S) (66%) ee _P = 99 (R) (34%)	198
65o	CH ₂ CH ₂ CN	CHMO _{Acineto} - <i>E. coli</i>	ee _S = 95 (S) (45%)	163
			ee _P = 97 (R) (50%)	

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses.

Figure 16. Kinetic resolution of 2-substituted cyclohexanones.

4.5. Asymmetric Transformations and Synthetic Applications

The real synthetic value of BVMO-mediated biotransformations lies in their extraordinary enantio-, regio-, and chemoselectivity. BVMO-mediated reactions have been exploited in kinetic resolutions, regiodivergent oxidations, deracemization of mesomeric compounds, and heteroatom oxidations in order to provide organic chemists versatile and enantiomerically pure intermediates. In addition to efforts in substrate screening, examples of chemoenzymatic synthesis of natural products using enzymatic BV oxidations will be discussed.

4.5.1. Kinetic Resolution of Racemic Compounds. The first indication for kinetic resolution of racemic starting materials was observed by Robert Shaw in 1966, when he cultured 11 bacteria in the presence of 2-heptylcyclopentanone (**63k**) (Figure 15).¹⁵¹ Extraction of the culture suspensions yielded in some cases lactones, and examination of the medium after biotransformations revealed them to be levorotatory. The author commented that this activity was ascribed to the produced lactone, since the applied cyclic ketone

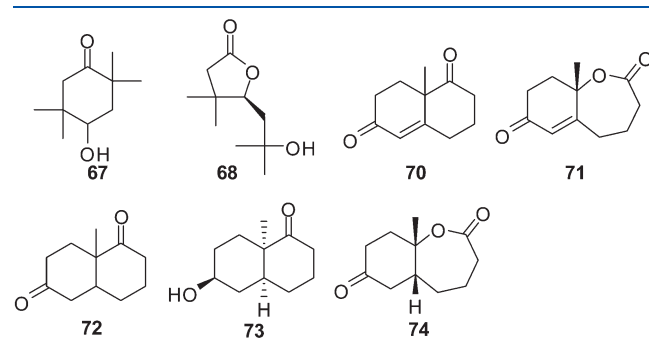
was racemic. The experimental proof that kinetic resolution actually took place was far from being watertight; however the possibility was very high.

In the early 1980s, Schwab's group studied the stereochemistry, enantioselectivity, and regioselectivity of the enzyme-catalyzed BV oxidations.¹⁵² CHMO from *Acinetobacter* was found to oxidize the (S)-enantiomer nearly twice as fast as the (R)-enantiomer of the "virtual racemate" **65b** consisting of (2R)-2-[methyl-²H₃] and (2S)-2-[methyl-¹³C]methylcyclohexanone. The initial rates of the oxidation were followed by ¹³C and ²H NMR spectroscopy (Figure 16).^{152b}

The first kinetic resolution on a preparative scale was reported by Azerad and co-workers, who could successfully resolve a derivative of 2,2,5,5-tetramethyl-4-hydroxy-cyclohexanone.¹⁵³ During attempted fungal reductions of the dione precursor, they isolated small amounts of a product previously obtained by reduction of one ketone functionality of 2,2,5,5-tetramethyl-1,4-cyclohexanedione followed by chemical BV oxidation.

When the 4-hydroxy-cyclohexanone derivative **67** was subjected to fungal suspensions under optimized fermentation conditions, the corresponding optically active rearranged (*S*)-hydroxy- γ -lactone **68** and nearly enantiopure starting material were recovered (Figure 17).¹⁵³

In 1980 Alphand, Archelas, and Furstoss described the kinetic resolution of substituted racemic cycloketones using BVMOs from two *Acinetobacter* sources on a preparative scale.¹⁵⁴ Oxidation of 2-undecylcyclopentanone (**63o**) with cell suspensions of either *Acinetobacter* NCIMB 9871 in the presence of the hydrolysis inhibitor tetraethylpyrophosphate or *Acinetobacter* TD 63 (devoid of lactone hydrolase) gave predominantly the (*S*)-isomer of 5-hexadecanolide (**64o**). The (*R*)-lactone **64o**, a pheromone isolated from the oriental hornet, *Vespa orientalis*, could easily be synthesized by chemical BV oxidation of the

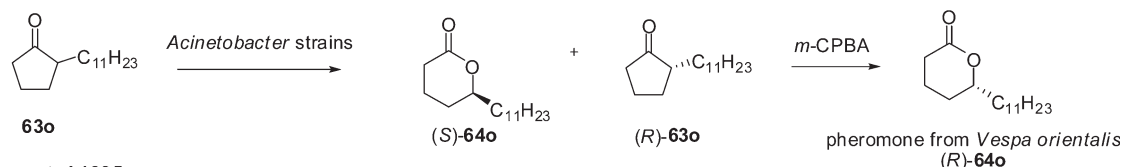


Substrate	Product	Biocatalyst	% ee (yield)	Reference
67	68	<i>Curvularia lunata</i> NRRL 2380	ee ₆₇ = 97 (42%) ee ₆₈ = > 95 (32%)	153
70	71	CHMO _{Acineto} - purified	ee ₇₀ = 80 (43%) ee ₇₁ = 99 (35%)	164
72	73 and 74	CHMO _{Acineto} - crude enzyme preparation	ee ₇₃ = 99 (32%) ee ₇₄ = 99 (22%)	164

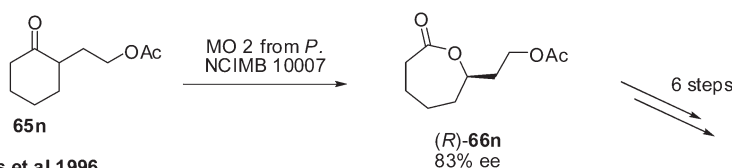
Figure 17. BVMO-mediated kinetic resolution of miscellaneous ketones.

remaining enantiomerically enriched ketone (*R*)-**63o**.¹⁵⁴ Subsequent studies by the same group described whole cell mediated BV oxidations of α -substituted cyclopentanones with varying side chain length (Figure 15).¹⁵⁵ In contrast to Furstoss's group who used whole cells for their biotransformations, Willets's group used partially purified MO fractions, the NADH-dependent fraction (also called MO1, a mixture of 2,5-DKCMO and 3,6-DKCMO), and the NADPH-fraction (also called MO2 consisting of OTEMO) from *P. putida* NCIMB 10007 for their potential use as biocatalysts for the resolution of 2-substituted cyclopentanones¹⁵⁶ and some 2-substituted cyclohexanones (Figure 16).¹⁵⁷ Of special interest was the enzymatic preparation of optically enriched lactone **66n**, which was converted in six steps into (*R*)-(+)-lipoic acid (**69**), a growth-promoting enzyme cofactor (Figure 18). Interestingly the same group reported that CPMO from *Pseudomonas* sp. NCIMB 9872 mediated oxidations of 2-(2'-acetoxyethyl)cyclohexanone (**65n**) as either whole cells or purified enzyme yielded the opposite enantiomer in moderate enantiomeric excess and allowed for a shortened formal synthesis of (*R*)-(+)-lipoic acid (**69**).¹⁵⁸ A direct comparison of whole cell (*Acinetobacter* TD63) and enzyme (purified NADPH-dependent MO from *P. putida* NCIMB 10007) mediated BV oxidations of 2-substituted cyclohexanones showed that both biocatalyst systems gave the same enantiomeric lactones, with *Acinetobacter* TD63 providing the highest *E*-values for all substrates (Figure 16).¹⁵⁹ The availability of new enzymatic BV reagents, such as CHMO from *Acinetobacter* NCIMB 9871 expressed in yeast and other recombinant BVMOs, simplified the experimental complexity for synthetic chemists and allowed for an extensive screening of possible substrates. The research groups of Kayser and Stewart used CHMO-producing "designer" yeast for the kinetic resolution of numerous cyclohexanones and cyclopentanones bearing different substituents at the 2-position,¹⁶⁰ and a collaboration of the groups of Kayser and Lau compared the synthetic utility of engineered yeast and *E. coli* overexpressing CHMO from *Acinetobacter* and CPMO for the kinetic resolution of racemic 2-substituted cyclopentanones.¹⁶¹ The most extensive study was undertaken by the group of Stewart and researchers from DuPont, who carried out a systematic investigation on five

Alphand and Furstoss 1990



Adger et al 1995



Bes et al 1996

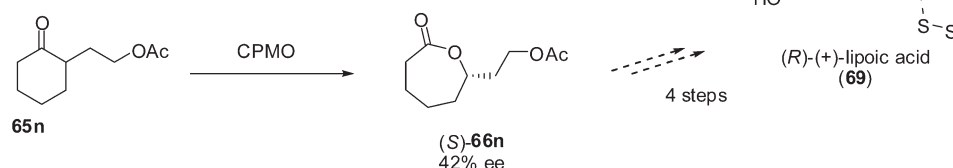
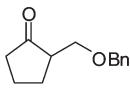
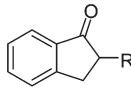


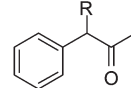
Figure 18. Applications of chiral synthons produced by BVMO-mediated kinetic resolution of racemic starting materials.



75



76a-d

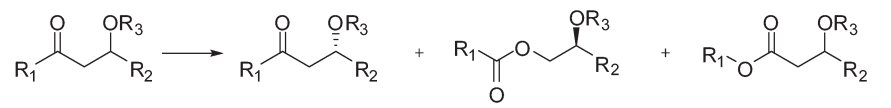


77a,b

Substrate	R	Biocatalyst ^a	Racemization Conditions	Yield %	% ee ^b	Reference
75		CHMO _{Acinet} - <i>E. coli</i>	pH 9	85	96 (R)	165
		CHMO _{Acinet} - <i>E. coli</i>	Lewatit MP62	89	96 (R)	166
76a	Me	PAMO (M446G)	pH 10; 5% hexanes	84 (conv.)	82 (R)	167
76b	Et	PAMO (M446G)	pH 10	91 (conv.)	72 (R)	167
76c	iso-Pr	PAMO (M446G)	pH 10; 5% MeOH	53 (conv.)	> 97 (R)	167
76d	n-Bu	PAMO (M446G)	pH 10; 5% MeOH	92 (conv.)	80 (R)	167
77a	Me	HAPMO	Dowex MWA-1	84 (conv.)	86 (S)	168
77b	Et	HAPMO	Dowex MWA-1	72 (conv.)	80 (S)	168

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses.

Figure 19. BVMO-mediated dynamic kinetic resolutions.



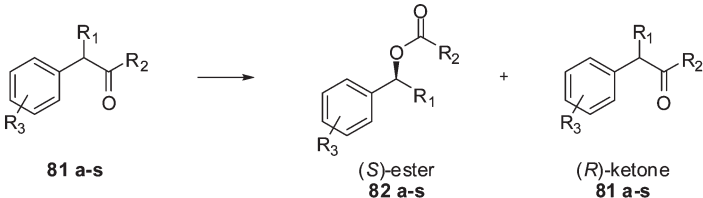
Substrate	R ₁	R ₂	R ₃	Biocatalyst ^a	E - value ^b	Reference
78a	Me	Bu	H	BmoF1 - <i>E. coli</i>	54 (S)	169
				MEKMO - purified	22 (R)	57
				crude extract HAPMO _{JD1}	1.3 (S)	53
				CHMO _{Acinet} - <i>E. coli</i>	156 (S)	171
				CPMO _{Coma} - <i>E. coli</i>	7 (S)	171
78b	Me	Pent	H	crude extract HAPMO _{JD1}	1.6 (S)	53
				CHMO _{Acinet} - <i>E. coli</i>	>200 (S)	171
				BmoF1 - <i>E. coli</i>	>200 (S)	171
78c	Me	Hex	H	BmoF1 - <i>E. coli</i>	55 (S)	169
				BmoF1 - <i>mutant</i>	92 (S)	170
				MEKMO - purified	3.8 (R)	57
				crude extract HAPMO _{JD1}	3.0 (S)	53
				CHMO _{Acinet} - <i>E. coli</i>	90 (S)	171
78d	Me	Hept	H	BmoF1 - <i>E. coli</i>	100 (S)	171
				crude extract HAPMO _{JD1}	1.1 (S)	53
				CHMO _{Acinet} - <i>E. coli</i>	4 (S)	171
78e	Me	Oct	H	CHMO _{Rhodo2} - <i>E. coli</i>	10 (S)	171
				BmoF1 - <i>E. coli</i>	41 (S)	169
				MEKMO - purified	4.2 (R)	57
78f	Me	Hex	Ac	CPMO _{Coma} - <i>E. coli</i>	>200 (R)	171
				CHMO _{Brevi2} - <i>E. coli</i>	31 (R)	171
78g	Me	Hex	CHO	CHMO _{Xantho} - <i>E. coli</i>	10 (S)	171
				CPMO - <i>E. coli</i>	>200 (S)	171
78h	Et	Bu	H	CHMO _{Acinet} - <i>E. coli</i>	35	171
				CPMO - <i>E. coli</i>	>200	171
				BmoF1 - <i>E. coli</i>	4 (64 re "abnormal")	171
78i	Et	Bu	H	CHMO _{Xantho} - <i>E. coli</i>	14	171
				CPMO - <i>E. coli</i>	35	171
				BmoF1 - <i>E. coli</i>	74 (40 re "abnormal")	171

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses.

Figure 20. BVMO-mediated resolution of aliphatic ketones.

substrates with nine different BVMOs; most notable among the biocatalysts was CDMO from *Rhodococcus* SC1, giving

exclusive oxidation of (S)-2-methyl cyclohexanone.¹⁶² Interestingly, CHMO from *Acinetobacter* catalyzed oxidations of



Substrate	R ₁	R ₂	R ₃	Biocatalyst ^a	<i>E</i> - value ^b	Reference
81a	Me	Me	H	BmoF1 - <i>E. coli</i>	43 (S)	172
				BVMO _{KT2440} - <i>E. coli</i>	12 (R)	172
				PAMO	188 (S)	173
				MEKMO - purified	3.1 (S)	57
				crude HAPMO _{JD1}	>200 (S)	53
81b	Et	Me	H	CHMO _{Acineto} - <i>E. coli</i>	4.5 (S)	172
				CPMO - <i>E. coli</i>	4.0 (R)	172
				PAMO	>200 (S)	173
				HAPMO	>200 (S)	173
81c	Me	Et	H	PAMO	>200 (S)	173
				HAPMO	117 (S)	173
81d	Et	Et	H	PAMO	179 (S)	173
				HAPMO	87 (S)	173
81e	Me	H	H	PAMO	26 (S)	173
				HAPMO	17 (S)	173
81f	Me	Me	<i>p</i> -MeO	PAMO	32 (S)	174
				HAPMO	136 (S)	174
81g	Me	Me	<i>p</i> -Et	PAMO	19 (S)	174
81h	Me	Me	<i>m</i> -Me	HAPMO	126 (S)	174
81i	Me	Me	<i>m</i> -MeO	PAMO (M446G)	52 (S)	174
81j	Me	Me	<i>p</i> -Br	PAMO (M446G)	44 (S)	174
81k	Me	Me	<i>p</i> -Cl	PAMO (M446G)	54 (S)	174
81l	Me	Me	<i>m</i> -Cl	HAPMO	63 (S)	174
81m	Me	Me	<i>m</i> -CF ₃	PAMO (M446G)	36 (S)	174
81n	Me	Me	<i>p</i> -NO ₃	PAMO (M446G)	121 (S)	174
81o	Bu	Me	H	PAMO (M446G)	15 (S)	174
81p	allyl	Me	H	PAMO	6 (S)	174
81q	Pr	Et	H	PAMO (M446G)	103 (S)	174
81r	Bu	Et	H	PAMO (M446G)	43 (S)	174
81s	allyl	Et	H	HAPMO	104 (S)	174

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses.

Figure 21. BVMO-mediated resolution of aryl aliphatic ketones.

α -substituted cyanocyclohexanones gave either a classical resolution with high enantioselectivity or a highly enantioselective regio-divergent oxidation depending on the length of the side chain.¹⁶³ Other BVMOs that have been used for the kinetic resolution of 2-substituted alkanones are CPDMO from *Pseudomonas* sp. HI-70,^{23b} displaying a similar substrate profile as CDMO, and CHMOs from *Rhodococcus* and *Xanthobacter* sp. ZLS.^{63,68} In addition to the work on monocyclic ketones, the kinetic resolution of racemic bicyclic diketones was reported by Ottolina and co-workers.¹⁶⁴ CHMO from *Acinetobacter* catalyzed the oxidation of diketones in a highly regio- and enantioselective manner. The presence of a dehydrogenase from crude CHMO preparations resulted in the formation of optically pure keto-alcohol 73 in addition to keto-lactone 74.¹⁶⁴

In summary, it can be said that BVMO-mediated oxidations are an excellent tool for the kinetic resolution of a wide range of α -substituted cyclic alkanones. Interestingly, nearly exclusively the (*S*)-ketones (depending on the substituent the priority numbering might be changed but the preferred chirality remains the same) are oxidized at a higher rate than the (*R*)-ketones by the BVMOs tested so far. CHMO-type biocatalysts show in most cases high to excellent enantioselectivities for the kinetic

resolution of α -substituted cyclic alkanones with a side chain length greater than C₂. CPDMO and CDMO are extremely effective in the resolution of 2-methyl cyclohexanone, and BVMOs from the camphor degradation pathway as well as CPMO give lower enantioselectivities in most cases than oxidations with CHMO from *Acinetobacter*.

4.5.2. Dynamic Kinetic Resolution. Any conventional kinetic resolution process only allows for a maximum yield of 50%, since only one isomer is converted and after the reaction both product and unchanged starting material have to be separated. In order to avoid these drawbacks, the group of Furstoss developed a BVMO-mediated dynamic kinetic resolution process.¹⁶⁵ Oxidation of 2-benzyloxymethyl cyclopentanone (75) with recombinant whole cells expressing CHMO from *Acinetobacter* NCIMB 9871 at a pH of 9 led to the isolation of 85% of the corresponding lactone in excellent ee (Figure 19).¹⁶⁵ Optimization of the reaction conditions and the use of a weakly basic anion exchange resin (Lewatit MP62) allowed for a 3-fold improvement of the applied substrate concentrations and the product was isolated in the same excellent enantioselectivity.¹⁶⁶

A mutant of PAMO (M446G) has been applied to the dynamic kinetic resolution of 2-alkyl-1-indanones **76a–d** under

Substrate	R	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
83a	Me	CHMO _{Acineto} - purified	80	>98 (-)	176
		CHMO _{Acineto} - yeast	83	>98 (-)	185
		CHMO _{Acineto} - <i>E. coli</i>	61	>98 (-)	186a
		CPMO - <i>E. coli</i>	68	46 (+) (R)	18
		CHMO _{brev1} - <i>E. coli</i>	65	>99 (-)	191
83b	OMe	CHMO _{Acineto} - purified	76	75 (-)	176
		CHMO _{Acineto} - <i>E. coli</i>	84	78 (+)	186a
		CPMO - <i>E. coli</i>	71	28 (+) (S)	187
83c	Et	CHMO _{Acineto} - purified	84	>98 (-)	179b
		CHMO _{Acineto} - yeast	74	>98 (-)	185
		CHMO _{Acineto} - <i>E. coli</i>	91	97 (-)	186a
83d	<i>iso</i> -Pr	CHMO _{Acineto} - purified	60	>98 (-)	179b
		CHMO _{Acineto} - yeast	60	>98 (-)	185
		CPMO _{Coma} - <i>E. coli</i>	79	33 (-) (S)	18
83e	<i>n</i> -Pr	CHMO _{Acineto} - purified	80	>98 (-)	179b
		CHMO _{Acineto} - yeast	63	92 (-)	185
		CPMO - <i>E. coli</i>	68	36 (S)	18
83f	<i>tert</i> -Bu	CHMO _{Acineto} - purified	17	>98 (-)	179b
		CPDMO - <i>E. coli</i>	68	99 (S)	23b
		CHMO _{Xantho} - <i>E. coli</i>	82	99 (-)	199
83g	CH ₂ OH	CHMO _{Acineto} - purified	80	>98 (-)	179b
83h	<i>n</i> -Bu	CHMO _{Acineto} - purified	70	52 (+)	179b
83i	OH	CHMO _{Acineto} - purified	30	9.6 (-)	179b
		CHMO _{Acineto} - <i>E. coli</i>	61	9.1 (-)	186a
		CPMO - <i>E. coli</i>	73	85 (+) (S)	187
83j	allyl	CHMO _{Acineto} - yeast	62	95 (-)	160b
		CPMO - <i>E. coli</i>	52	42 (+) (S)	18
83k	Br	CHMO _{Acineto} - <i>E. coli</i>	63	97 (-)	186a
		CPMO - <i>E. coli</i>	70	64 (+) (R)	187
83l	I	CHMO _{Acineto} - <i>E. coli</i>	60	97 (-)	186a
		CPMO - <i>E. coli</i>	65	82 (+) (R)	187
		CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (-)	199
83m	Cl	CHMO _{Acineto} - <i>E. coli</i>	15	93 (-)	187
		CPMO - <i>E. coli</i>	83	64 (+)	187
83n	COOEt	CHMO _{Acineto} - <i>E. coli</i>	56	95 (-) (S)	187
		CPMO - <i>E. coli</i>	64	34 (+) (R)	187
		CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	98 (-)	199
83o	OEt	CPMO - <i>E. coli</i>	90	37 (-)	187
83p	Oallyl	CPMO - <i>E. coli</i>	80	45 (+) (S)	187
83q	OBn	CPMO - <i>E. coli</i>	95	75 (-) (S)	187
83r	OAc	CPMO - <i>E. coli</i>	81	5 n. d.	187
83s	Ph	CHMO _{Xantho} - <i>E. coli</i>	88	98 (-)	199

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation or absolute configuration of product is given in parentheses; ^c n. d. = not determined.

Figure 22. Desymmetrization of 4-substituted cyclohexanones.

basic conditions by the group of Gotor. In some cases, addition of organic solvents, such as 5% *n*-hexane, led to an improvement in yield and enantioselectivity of the obtained 3-alkyl-3,4-dihydroisocoumarins.¹⁶⁷ The same group used HAPMO from *Pseudomonas fluorescens* ACB at a basic pH in the presence of an anion exchange resin for the dynamic kinetic resolution of racemic α -substituted benzyl ketones.¹⁶⁸ Different anion exchange resins were tested, and optimization of the reaction conditions led in some cases to conversions of more than 90% in good enantioselectivities.

4.5.3. Kinetic Resolution of Aliphatic and Aryl-Aliphatic Ketones. In addition to the work on cyclic ketones, racemic

aliphatic ketones have been applied to BVMO-mediated resolutions. In 2006, Kirschner and Bornscheuer reported the regio- and enantioselective kinetic resolution of 4-hydroxy-2-aliphatic ketones (78a, 78c, 78e) by the action of a recombinant BVMO from *P. fluorescens* DSM 50106.¹⁶⁹ Ketones were oxidized to the corresponding (*S*)-hydroxyalkyl acetates 79 with good enantioselectivities (*E* = 55). Directed evolution of the native enzyme and optimization of the reaction conditions led to an improvement of the enantioselectivity (*E* \approx 92).¹⁷⁰ The same substrates were oxidized by a BVMO from *P. veronii* MEK700 to give the enantiomerically enriched protected (*S*)-diols, but in only low selectivity (*E* = 22),⁵⁷ and by a BVMO from *P. putida* JD1 to give

86 a-h **87 a-h** **88a, 88e**
 if R₁ or R₂ = OH

Substrate	R ₁	R ₂	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
86a	OH	H	CHMO _{Acineto} - purified	88	>98 (-)	176
			CHMO _{Acineto} - <i>E. coli</i>	77	>98 (-)	190
			CPMO - <i>E. coli</i>	n. c.		190
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>95 (-)	199
86b	H	H	CHMO _{Acineto} - purified	73	>98 (-)	176
			CHMO _{Brev1} - <i>E. coli</i>	61	97 (-)	191
			CHMO _{Brev2} - <i>E. coli</i>	56	99 (+)	191
			CHMO _{Acineto} - <i>E. coli</i>	65	>99 (-)	190
			CPMO - <i>E. coli</i>	58	91 (+)	190
86c	=O	=CH ₂	CHMO _{Acineto} - purified	25	>98 (-)	176
86d			CHMO _{Brev1} - <i>E. coli</i>	61	> 99 (+)	191
	CHMO _{Brev2} - <i>E. coli</i>	56	> 99 (-)	191		
	CHMO _{Acineto} - <i>E. coli</i>	54	92 (+)	190		
	CPMO - <i>E. coli</i>	63	99 (-)	190		
86e	H	OH	CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (+)	199
			CHMO _{Acineto} - <i>E. coli</i>	80	96 (+)	190
			CPMO - <i>E. coli</i>	n. c.		190
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>95 (+)	199
86f	Cl	H	CHMO _{Acineto} - <i>E. coli</i>	53	99 (-)	190
			CPMO - <i>E. coli</i>	n. c.		190
86g	H	Cl	CHMO _{Acineto} - <i>E. coli</i>	40	>99 (-)	190
			CPMO - <i>E. coli</i>	n. c.		190
86h	cyclo-CH ₂ CH ₂		CHMO _{Acineto} - <i>E. coli</i>	57	>99 (+)	190

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses; ^c n. c. = no conversion.

Figure 23. Desymmetrization of 3,5-dimethyl-substituted cyclohexanones.

Substrate	R	= / -	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
90a	H	=	CHMO _{Acineto} - purified	62	80 (+)	179a
90b	Me	=	CHMO _{Acineto} - purified	70	>98 (+)	179a
90c	Et	=	CHMO _{Acineto} - purified	83	93 (+)	179a
90d	-CH ₂ OCH ₂ -	=	CHMO _{Acineto} - purified	74	>98 (+)	179a
90e	-(CH ₂) ₃ -	=	CHMO _{Acineto} - purified	80	97 (+)	179a
90f	-(CH ₂) ₄ -	=	CHMO _{Acineto} - purified	78	>98 (-)	179a
90g	-(CH ₂) ₅ -	=	CHMO _{Acineto} - purified	57	>98 (+)	179a
90h	-(CH ₂) ₆ -	=	CHMO _{Acineto} - purified	55	87 (+)	179a
90i	-(CH ₂) ₂ -	-	CHMO _{Brev1} - <i>E. coli</i>	72	96 (-)	197
			CPMO - <i>E. coli</i>	56 conv	86 (+)	197
90j	-CH ₂ OCH ₂ -	-	CHMO _{Acineto} - <i>E. coli</i>	53	92 (-)	195b
			CPMO - <i>E. coli</i>	49	71 (+)	195b
90k	-(CH ₂) ₃ -	-	CHMO _{Rhodo1} - <i>E. coli</i>	63	99 (-)	195b
			CHMO _{Brev2} - <i>E. coli</i>	67	92 (+)	195b
90l	-(CH ₂) ₄ -	-	CHMO _{Rhodo2} - <i>E. coli</i>	58	99 (-)	195b
			CHMO _{Brev2} - <i>E. coli</i>	78	94 (+)	195b

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses.

Figure 24. Desymmetrization of substituted bicyclo[2.2.1]heptanones.

nearly racemic hydroxyalkyl acetates (Figure 20).⁵³ Twelve BVMOs from different bacterial origin were tested as suitable biocatalysts

for the regio- and enantioselective oxidation of linear aliphatic β -hydroxyketones.¹⁷¹ The best enantioselectivities were obtained

92 a-m **93 a-m**

Substrate	R	Biocatalyst	Yield (%)	% ee	Reference
92a	Bu	<i>Acinetobacter</i> NCIMB 9871	68	17 (S)	181
		MO1 - crude enzyme	100 conv	69 (R)	181
		CHMO _{Brev1} - <i>E. coli</i>	65	>99 (S)	194
92b	<i>iso</i> -Bu	<i>Acinetobacter</i> NCIMB 9871	56	84 (S)	181
		MO1 - crude enzyme	78 conv	91 (R)	181
		MO2 - crude enzyme	97 conv	85 (R)	181
		CHMO _{Brev1} - <i>E. coli</i>	30	>99 (S)	194
92c	Bn	<i>Acinetobacter</i> NCIMB 9871	57	82 (S)	181
		HAPMO - <i>E. coli</i>	26	44 (R)	200
		CHMO _{Arthro} - <i>E. coli</i>	56	93 (S)	194
92d	piperonyl	<i>Acinetobacter</i> NCIMB 9871	83	95 (S)	181
		<i>C. echinulata</i> NRRL 3655	70	99 (S)	182a
		HAPMO - <i>E. coli</i>	63	66 (R)	200
		CHMO _{Xantho} - <i>E. coli</i>	>90 conv	99 (S)	199
92e	CH ₂ OBn	<i>Acinetobacter</i> NCIMB 9871	89	55 (S)	181
		MO1 - crude enzyme	74	74 (S)	181
		MO2 - crude enzyme	95 conv	90 (R)	181
		<i>C. echinulata</i> NRRL 3655	74	98 (R)	182a
92f	Ph	<i>Acinetobacter</i> NCIMB 9871	70	43 (R)	182a
		<i>C. echinulata</i> NRRL 3655	71	>98 (R)	182b
		HAPMO - <i>E. coli</i>	12	92 (S)	200
		CHMO _{Brev1} - <i>E. coli</i>	73	98 (R)	75
92g	<i>p</i> -F-Ph	<i>Acinetobacter</i> NCIMB 9871	89	19	182a
		<i>C. echinulata</i>	80	>98	182a
92h	<i>p</i> -Cl-Ph	<i>Acinetobacter</i> NCIMB 9871	88	85 (S)	182a
		<i>C. echinulata</i> NRRL 3655	30	>98 (R)	182a
		<i>Acinetobacter</i> TD63	15	89 (S)	182a
		CHMO _{Rhodo2} - <i>E. coli</i>	63	95 (S)	194
92i	<i>p</i> -Me-Ph	<i>Acinetobacter</i> NCIMB 9871	73	91 (S)	182a
		<i>Acinetobacter</i> TD63	61	93 (S)	182a
92j	<i>m</i> -MeO-Bn	<i>Acinetobacter</i> NCIMB 9871	83	96 (S)	182a
		<i>C. echinulata</i> NRRL 3655	68	77 (S)	182a
		<i>Acinetobacter</i> TD63	94	94 (S)	182a
		HAPMO - <i>E. coli</i>	42	56 (R)	200
		CHMO _{Rhodo1} - <i>E. coli</i>	60	98 (S)	194
		CHMO _{Xantho} - <i>E. coli</i>	>90 conv	97 (S)	199
92k	CH ₂ O- <i>tert</i> -Bu	<i>Acinetobacter</i> NCIMB 9871	43	89	182a
		<i>C. echinulata</i> NRRL 3655	25	>98	182a
92l	<i>p</i> -MeO-Bn	CHMO _{Arthro} - <i>E. coli</i>	89	97 (S)	194
		CHMO _{Brev1} - <i>E. coli</i>	73	26 (S)	194
92m	3,4,5-(MeO) ₃ -Bn	CHMO _{Arthro} - <i>E. coli</i>	72	94 (S)	194
		CHMO _{Brev1} - <i>E. coli</i>	72	78 (R)	194
		CHMO _{Xantho} - <i>E. coli</i>	>90 conv	95 (S)	199

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses.

Figure 25. Desymmetrization of 3-substituted cyclobutanones.

for the kinetic resolution of 4-hydroxy-2-ketones (e.g., **78a–c**) ($E > 100$), whereas the formation of significant amounts of the “abnormal” lactones (**80**) derived from 5-hydroxy-3-ketones (**78h**, **78i**) was observed when a BVMO from *P. fluorescens* DSM 50106 was used.¹⁷¹ For a discussion on the regioselectivity of BVMO-mediated oxidations, please see section 4.5.5 of this review.

Although screened as suitable substrates in biochemical studies,⁵⁰ aryl-aliphatic ketones were first used in enantioselective BVMO-mediated kinetic resolutions in 2007. Four different

BVMOs (CHMO_{Acinetor}, CPMO, and BVMOs originating from *P. fluorescens* DSM 50106 and *P. putida* KT2440), expressed recombinantly in *E. coli*, were applied to the resolution of two racemic 3-phenyl-2-ketones.¹⁷² High enantioselectivities ($E = 43$) were observed for the kinetic resolution of 3-phenyl-2-butanone (**81a**) with the BVMO from *P. fluorescens* DSM 50106, and reactions with the BVMO from *P. putida* KT2440 showed an opposite (*R*)-enantiopreference. Purified enzyme preparations of PAMO and HAPMO were used for the oxidation of four racemic

97 a-h **98 a-h**

Substrate	R ₁	R ₂	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
97a	Me	Me	CHMO _{Acineto} - <i>E. coli</i>	61	n. a.	186a
97b	Et	Me	CHMO _{Acineto} - <i>E. coli</i>	91	75 (-)	186a
			CHMO _{Arthro} - <i>E. coli</i>	62	88 (-)	193
			CPMO - <i>E. coli</i>	56	21 (-)	193
			CHMO _{Brevi2} - <i>E. coli</i>	78	43 (-)	193
97c	Et	Et	CHMO _{Acineto} - <i>E. coli</i>	60	n. a.	186a
97d	cyclo-CH ₂ CH ₂		CHMO _{Acineto} - <i>E. coli</i>	74	n. a.	186a
97e	Et	OH	CHMO _{Acineto} - <i>E. coli</i>	54	94 (-)	186a
			CPMO - <i>E. coli</i>	52	42 (+) (S)	18
97f	Me	<i>n</i> -Propyl	CPMO - <i>E. coli</i>	89	n. d. (-)	18
97g	Me	OH	CHMO _{Acineto} - <i>E. coli</i>	59	86 (-)	75
			CHMO _{Brachy} - <i>E. coli</i>	48	97 (-)	75
			CPMO _{Corna} - <i>E. coli</i>	54	76 (+)	75
			CHMO _{Brevi2} - <i>E. coli</i>	37	61 (+)	75
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (-)	199
97h	Ph	Me	CHMO _{Xantho} - <i>E. coli</i>	60	95 (-)	199

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses; ^c n. a. = not applicable; ^d n. d. = not determined.

Figure 26. Desymmetrization of 4,4-disubstituted cyclohexanones.

99 a-l **100 a-l**

Substrate	R	X	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
99a	H	S	CHMO _{Acineto} - <i>E. coli</i>	48	n. a.	186b
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	n. a.	199
99b	H	O	CHMO _{Acineto} - <i>E. coli</i>	79	n. a.	186b
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	n. a.	199
			CHMO _{Brevi1} - <i>E. coli</i>	76	n. a.	196
			CPMO - <i>E. coli</i>	75	n. a.	196
99c	H	NMe	CHMO _{Acineto} - <i>E. coli</i>	50	n. a.	186b
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	n. a.	199
99d	H	Nallyl	CHMO _{Acineto} - <i>E. coli</i>	10	n. a.	186b
99e	H	NAc	CHMO _{Acineto} - <i>E. coli</i>	39	n. a.	186b
99f	H	NCO ₂ Me	CHMO _{Acineto} - <i>E. coli</i>	40	n. a.	186b
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	n. a.	199
99g	Me	O	CHMO _{Acineto} - <i>E. coli</i>	80	>99.5 (-)	186c
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (-)	199
			CPMO - <i>E. coli</i>		traces	196
99h	ethyl	O	CHMO _{Acineto} - <i>E. coli</i>	90	>99.5 (-)	186c
99i	<i>n</i> -Pr	O	CHMO _{Acineto} - <i>E. coli</i>	19	98 (-)	186c
99j	<i>iso</i> -Pr	O	CHMO _{Acineto} - <i>E. coli</i>		traces	186c
99k	Butyl	O	CHMO _{Acineto} - <i>E. coli</i>		n. c.	186c
99l	vinyl	O	CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (-)	199
			CHMO _{Acineto} - <i>E. coli</i>	40	>99 (-)	196
			CHMO _{Brevi1} - <i>E. coli</i>	64	98 (-)	196

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses; ^c n. a. = not applicable; ^d n. c. = no conversion.

Figure 27. Desymmetrization of heteroatom-containing cyclohexanone derivatives.

101 a-h **102 a-h**

Substrate	X	R	Biocatalyst ^a	Yield (%)	% ee ^a	Reference
101a	-CH=CH-	-	CHMO _{Acineto} - <i>E. coli</i>	33	5 (-)	188
			CPMO - <i>E. coli</i>	76	> 99 (+)	188
			CHMO _{Brev1} - <i>E. coli</i>	10	71	191
			CHMO _{Brev2} - <i>E. coli</i>	92	94 (+)	191
			CHMO _{Brachy} - <i>E. coli</i>	56	85 (-)	75
101b	-CH ₂ -CH ₂ -	-	CHMO _{Acineto} - <i>E. coli</i>	21	3 (-)	188
			CPMO - <i>E. coli</i>	83	99 (+)	188
101c	-CH ₂ -	H	CHMO _{Acineto} - <i>E. coli</i>	50	89 (-)	75
			CPMO - <i>E. coli</i>	89	9 (+)	75
			CHMO _{Brachy} - <i>E. coli</i>	71	91 (-)	75
101d	-CH-	MeO <i>endo</i>	CHMO _{Acineto} - <i>E. coli</i>	24	9 (-)	189b
			CPMO - <i>E. coli</i>	81	34 (+)	189b
101e	-CH-	MeO <i>exo</i>	CHMO _{Acineto} - <i>E. coli</i>	40	96 (-)	189b
			CPMO - <i>E. coli</i>	75	11 (-)	189b
101f	-CH-	Cl <i>endo</i>	CHMO _{Acineto} - <i>E. coli</i>	75	80 (-)	189b
			CPMO - <i>E. coli</i>	79	60 (-)	189b
101g	-CH-	Cl <i>exo</i>	CHMO _{Acineto} - <i>E. coli</i>	78	>99 (-)	189b
			CPMO _{Corn} - <i>E. coli</i>	92	>99 (+)	189b
			CHMO _{Brev1} - <i>E. coli</i>	55	>99 (-)	191
			CHMO _{Brev2} - <i>E. coli</i>	59	>99 (+)	191
101h	-C-	=CH ₂	CHMO _{Acineto} - <i>E. coli</i>	43	61 (-)	189b
			CPMO - <i>E. coli</i>	85	41 (+)	189b
			CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	>99 (-)	199

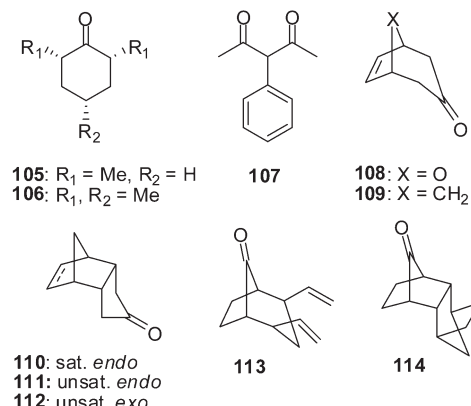
^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses.

Figure 28. Desymmetrization of 6,5- and 5,5-bicyclic ketones.

aryl-aliphatic ketones and 2-phenylpropionaldehyde (**81e**) by the group of Gotor.¹⁷³ Optimization of the reaction conditions led to excellent enantioselectivities for all ketone substrates in contrast to the oxidation of the propionaldehyde derivative, which was resolved in rather low enantioselectivities (*E* = 26). In a more detailed study, the same group applied purified PAMO, the PAMO mutant M446G, and HAPMO to the kinetic resolution of a wide range of 3-phenylbutanones substituted at the aromatic moiety, **81f–n**, and α -substituted benzylketones with different alkyl chain lengths, **81b**, **81d**, **81o–81s** (Figure 21).¹⁷⁴ Two other BVMOs (BmoF1 from *P. veronii* MEK700⁵⁷ and HAPMO from *P. putida* JD1⁵³) have been used for the resolution of 3-phenyl-2-butanone (**81a**), with HAPMO giving excellent enantioselectivity (*E* = 200).

4.5.4. Desymmetrization of Prochiral Ketones. An incredible amount of research has been devoted to the establishment of enantioselective routes to the synthesis of enantiomerically pure chemicals. Enzyme-catalyzed desymmetrizations of prochiral or meso substrates have been recognized as a powerful tool and have been reviewed lately by Gotor and co-workers.¹⁷⁵ It is no surprise that BVMOs have been screened for their potential use in desymmetrizations of ketones. In 1988, Taschner and Black reported the first series of enzymatic BV oxidations of mesomeric cyclohexanones.¹⁷⁶ Purified CHMO from *Acinetobacter* NCIMB 9871 was shown to be an excellent biocatalyst for the enantioselective preparation of lactones **83a**, **83b**, and **86a–c** (Figure 22, Figure 23). Of special interest was the enzymatic oxidation of *cis*-3,5-dimethyl cyclohexanone (**86b**), which allowed for an efficient synthesis of the C₁₁–C₁₆ subunit of ionomycin **89**, a naturally occurring polyether antibiotic (Figure 30).¹⁷⁷ In

comparison, the same intermediate was prepared chemically in seven steps by Evans and 25 steps by Hanessian.¹⁷⁸ Taschner and co-workers extended this methodology to the desymmetrization of a series of substituted bicyclo[2.2.1]hept-2-en-7-ones (**90a–h**, Figure 24) and 4-substituted cyclohexanones **83c–i**.¹⁷⁹ For each of the bicyclic substrates, the corresponding lactones were isolated in good yield and excellent ee values.^{179a} Similar results were obtained for the CHMO-mediated oxidation of 4-substituted cyclohexanones bearing aliphatic C₂ to C₃ substituents in the 4-position, while a significant decrease in yield was observed for 4-*tert*-butyl cyclohexanone (**83f**); the *n*-butyl derivative **83h** was oxidized efficiently but in a lower enantioselectivity. Prochiral cyclohexanones bearing hydroxyl functionalities were found to rearrange to the thermodynamically favored C₅ lactones **85i**, **88a**, and **88e** upon initial CHMO_{Acineto}-mediated oxidation. The potential synthetic value of these rearranged lactones was recognized by Taschner and Aminbhavi as suitable intermediates for the synthesis of the C₅–C₁₀ subunit of 3-acyltetramic acid antibiotics.¹⁸⁰ Based on the seminal work of Taschner, 3-substituted cyclobutanones **92** were subjected to the catalytic activity of either whole cells of *Acinetobacter* NCIMB 9871 or enzyme preparations of MO1 and MO2 from the camphor-degrading strain *P. putida* NCIMB 10007 (Figure 25).¹⁸¹ Oxidation of the prochiral ketones gave 3-substituted γ -lactones in moderate to excellent yield and moderate enantioselectivity. Interestingly in some cases, the MOs from *Acinetobacter* NCIMB 9871 and *P. putida* NCIMB 10007 showed complementary stereoselectivities. The rather low ee values from the biotransformations with bacterial MOs initiated a screening for alternative biocatalysts, resulting in the discovery of *Cunninghamella echinulata* NRRL



105: R₁ = Me, R₂ = H
106: R₁, R₂ = Me

107

108: X = O
109: X = CH₂

110: sat. *endo*
111: unsat. *endo*
112: unsat. *exo*

113

114

Substrate	Biocatalyst ^a	Yield (%)	% ee ^b	Reference
105	CHMO _{Acinet} - purified	27	>98 (-)	176
	CPDMO - <i>E. coli</i>	74 conv	99	23b
106	CHMO _{Acinet} - purified	100 conv	48 (+)	198
107	PAMO - purified	88 conv	82 (R)	201
108	CPMO - <i>E. coli</i>	53	95 (+)	75
	CHMO _{Brevi2} - <i>E. coli</i>	19	93 (+)	75
109	CHMO _{Xantho} - <i>E. coli</i>	71	>99 (-)	199
110	CHMO _{Arthro} - <i>E. coli</i>	63	95 (+)	195a
	CHMO _{Brevi2} - <i>E. coli</i>	62	23 (-)	195a
111	CHMO _{Rhod1} - <i>E. coli</i>	6 conv	92 (+)	195a
	CPMO - <i>E. coli</i>	68	74 (-)	195a
	CHMO _{Xantho} - <i>E. coli</i>	< 50 conv	94 (+)	199
112	CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	43 (+)	199
113	CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	98 : 2 <i>syn/anti</i>	199
114	CHMO _{Acinet} - <i>E. coli</i>	51	98 (-)	195b
	CHMO _{Xantho} - <i>E. coli</i>	> 90 conv	92 (-)	199

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation of product is given in parentheses.

Figure 29. Desymmetrization of miscellaneous compounds.

3655 possessing significant BVMO activity. A series of cyclobutanones was shown to be transformed by *C. echinulata* in excellent enantioselectivities and good yield.¹⁸² The obtained γ -butyrolactones **93e** and **93f** were further used as starting materials for the total synthesis of (*R*)- β -proline (**94**), (*S*)- β -proline (**95**),¹⁸³ and (*R*)-baclofen (**96**) (Figure 30).¹⁸⁴

Desymmetrization of 4-mono- and 4,4-disubstituted cyclohexanones (Figure 26), as well as prochiral perhydropyranones (Figure 27) was used by the groups of Kayser, Stewart, and Mihovilovic to showcase the utility, efficiency, and enantioselectivity of cloned CHMO_{Acinet} in baker's yeast^{160c,185} and *E. coli*.¹⁸⁶

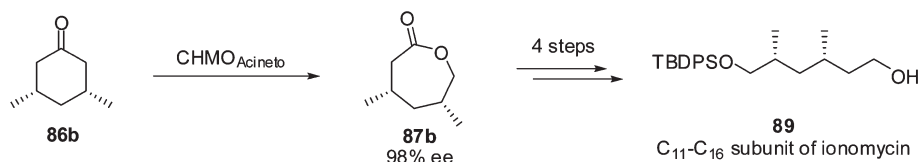
The first indications for an enantiodivergent trend in the oxidation of symmetrical ketones with BVMOs, namely, CHMO_{Acinet} and CPMO, were observed nearly simultaneously by the groups of Lau and Mihovilovic in 2002. Substrate acceptance studies with CPMO and comparison with data from CHMO_{Acinet} revealed that for some substrates (mono and disubstituted cyclohexanones) opposite enantiomers were obtained as products.^{18,187} At the same time, Mihovilovic and co-workers applied whole cells over-expressing CHMO_{Acinet} and CPMO to the desymmetrization of fused bicycloketones (6,5- and 5,5-bicyclic ketones). Biotransformations of substituted bicyclo[4.3.0]ketones **101a** and **101b** using recombinant CPMO expressing whole cells gave lactones in excellent ee values, whereas rather low ee values of the opposite enantiomers were produced by cells expressing CHMO (Figure 28).¹⁸⁸ It has to be mentioned that these lactones are of

high synthetic value, since they represent key intermediates for the synthesis of indole alkaloids such as (–)-alloyohimbane (**103**) and (–)-antirrhine (**104**). A more distinct complementary behavior of CPMO and CHMO was observed during the conversion of substituted bicyclo[3.3.0]ketones; for substrate **101g** both enantiopure products could be obtained depending on the BVMO applied.¹⁸⁹ A distinct difference in substrate acceptance, and in a few cases in enantioselectivity, was observed for the oxidation of symmetrical 3,5-dimethylcyclohexanones bearing various functionalities with whole cells expressing CHMO and CPMO (Figure 23).¹⁹⁰ Two MOs from *Brevibacterium* performed enantiodivergent biotransformations on mono-, di-, and polysubstituted cyclohexanones, as well as fused bicyclic ketones, giving access to both enantiomers in good to excellent ee values.¹⁹¹ A systematic investigation of the desymmetrization of 4-substituted cyclohexanones with eight cloned BVMOs originating from *Brevibacterium* sp. (two BVMOs), *Acidovorax* CHX, *Acinetobacter* SE19, *Arthrobacter* BP2, *Rhodococcus* phi1, *Rhodococcus* phi2, and *Rhodococcus* SC1, was conducted by Stewart and co-workers, and the results were compared with the ones obtained for CHMO (*Acinetobacter* NCIMB 9871) mediated transformations.¹⁶² The newly cloned biocatalysts showed nearly exclusively (*S*)-selectivity similar to CHMO and for each substrate (Me, Et, *n*-Pr, *i*-Pr, *n*-Bu) at least one biocatalyst could be identified to yield the enantiopure (*S*)-lactone. For 4-butyl-cyclohexanone some catalysts gave preferentially (*R*)-lactones, however not in synthetically useful enantiomeric purities.¹⁶²

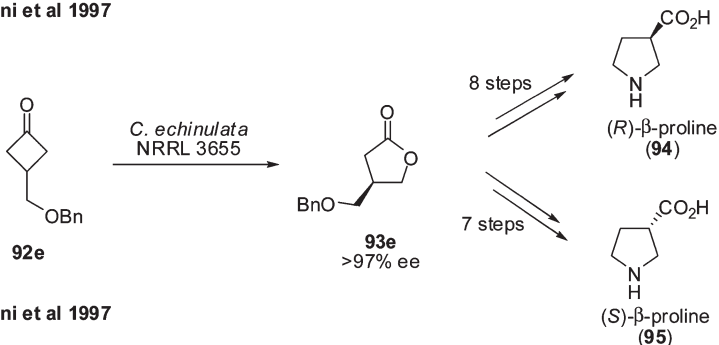
Based on these previous reports, Mihovilovic and co-workers applied a similar library as previously used by Stewart, namely, CHMO from *Acinetobacter* NCIMB 9871, two BVMOs from each *Brevibacterium* and *Rhodococcus*, CHMO from *Brachymonas* and *Arthrobacter*, and CPMO from *Comamonas* sp., to study in detail the substrate acceptance and stereopreference of these BVMOs. Both in the biocatalytic performance and in the phylogenetic analysis, two distinct clusters were observed. One cluster consisting of BVMOs from *Acinetobacter* NCIMB 9871, *Brevibacterium* (one out of two), *Rhodococcus*, *Brachymonas*, and *Arthrobacter* was named CHMO after the prototype BVMO from *Acinetobacter* NCIMB 9871. CPMO from *Comamonas* sp. and one BVMO from *Brevibacterium* formed the second cluster.⁷⁵ Of special interest to the group of Mihovilovic was the conversion of 8-oxabicyclo[3.2.1]oct-6-en-3-one (**108**), which was only converted with CPMO-type enzymes (Figure 29).⁷⁵ Optimization of the fermentation conditions and the use of a substrate feeding and product removal strategy allowed for isolation of the desired lactone in 70% yield at high substrate concentrations (5 g/L) with whole cells expressing CPMO. The produced lactone **115** facilitated an efficient formal synthesis of the tetrahydrofuran-based natural products (+)-*trans*-kumausyne (**119**), analogues of goniofurone such as compound **120**, and (+)-showdomycin (**121**).¹⁹²

The same biocatalyst library was further used to screen for enantioselective biooxidations of 4,4-disubstituted cyclohexanones and cyclohexenones (Figure 26),¹⁹³ substituted cyclobutanones (Figure 25),¹⁹⁴ bridged cycloketones (Figure 24),¹⁹⁵ and perhydropyran-type ketones (Figure 27).¹⁹⁶ For each substrate class, two BVMO families distinct in their biocatalytic behavior were identified, and in several cases, antipodal lactones could be isolated in excellent enantioselectivities. These observations were in accordance with Mihovilovic's previous classification of the eight BVMOs in a CPMO- and CHMO-family.⁷⁵ Interestingly, unsaturated ketones were not converted by whole cells expressing BVMOs; however in some cases, the starting materials were

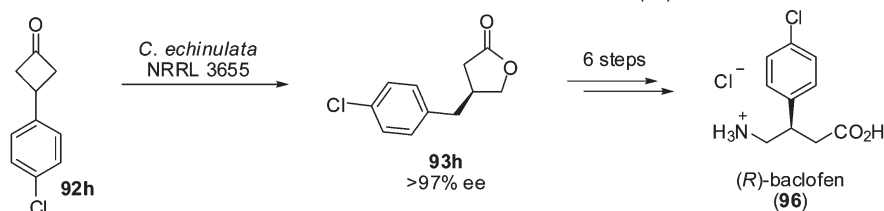
Taschner and Chen 1991



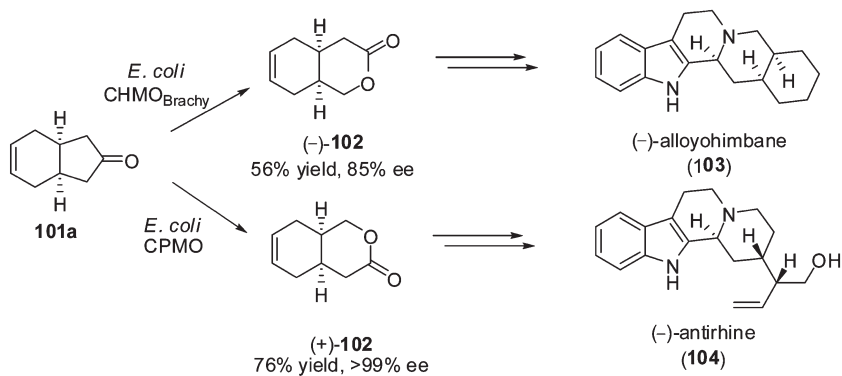
Mazzini et al 1997



Mazzini et al 1997



Mihovilovic et al 2002 and 2005



Mihovilovic et al 2006

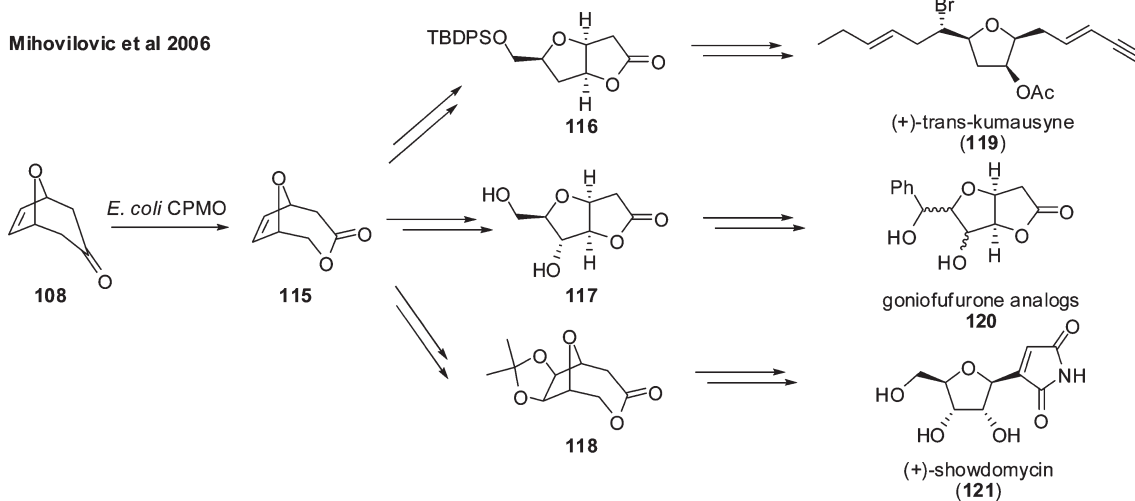
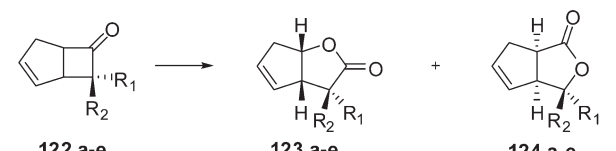


Figure 30. Applications of chiral synthons derived from BVMO-mediated desymmetrization reactions.



Substrate	R ₁	R ₂	Biocatalyst	Yield (%)	Ratio 123 : 124	% ee "normal" ^a	% ee "abnormal" ^a	Reference
122a	H	H	<i>Acinetobacter</i> TD63	86	51:49	>97 (1S, 5R)	>97 (1R,5S)	203
			<i>C. echinulata</i> NRRL 3655	30	0:100	-	>98 (1R,5S)	204a
			2,5-DKCMO	100 conv	1:1.3	100 (1R,5S)	82 (1S, 5R)	209f
			3,6-DKCMO	30 conv	1:1.3	72 (1R,5S)	10 (1S, 5R)	209f
			MO2	100 conv	72:28	35 (1S, 5R)	95 (1R,5S)	209f
			CPMO	61	97:3	0 (1S, 5R)	>99 (1R,5S)	212b
122b	Me	H	<i>Acinetobacter</i> NCIMB 9871	55	1:1	>95 (1S,5S)	>95 (1R,5S)	209a
			<i>P. putida</i> NCIMB 10007	63	1:1	80 (1R,5R)	>95 (1S,5R)	156
122c	Me	Me	<i>Acinetobacter</i> NCIMB 9871	63	0:100	-	29	209a
			M01	100 conv	n. r.	80 (1R,5S)	95 (1S, 5R)	209c
			M02	100 conv	n. r.	60 (1R,5S)	95 (1S, 5R)	209c
122d	Pr	H	<i>Xanthobacter</i>	100 conv	43:57	>99 (1S,5S)	>99 (1R,5S)	209e
			<i>P. putida</i> AS1	70	37:33	>99	>99	209d
122e	CH ₂ CH ₂ Cl	H	<i>Acinetobacter</i> NCIMB 9871	100 conv	27:73	82	97	202

^a absolute configuration of product is given in parentheses; ^b n. r. = not reported.

Figure 31. Regiodivergent oxidations of bicyclo[3.2.0]heptanone derivatives.

first reduced to the saturated ketones by the action of a reductase activity from the *E. coli* host strain and subsequently oxidized by the MO.¹⁹³ Enantiomerically pure bicyclo[4.2.0]octanes, common frameworks of natural products, were synthesized by a fragmentation reaction of tricyclo[4.2.1.0^{2,5}]nonan-9-one (**91i**, Figure 24). Different strategies for the enantioselective ring-opening reaction were investigated, and whole cell mediated BV oxidations (especially the BVMOs from *Brevibacterium*) were found to be the most successful, giving both antipodal products in high yields and excellent enantioselectivities depending on the source of biocatalyst.¹⁹⁷

CHMO_{Acineto}-mediated oxidation of 2,4,6-trimethylcyclohexanone (**105**) was reported by Vogel and co-workers as an alternative route for the enantioselective synthesis of the corresponding lactone; however the product, which has been used as a precursor for the synthesis of (–)-lardolure, was isolated only in moderate ee.¹⁹⁸ CPDMO from *Pseudomonas* sp. HI-70^{23b} and CHMO from *Rhodococcus* sp. HI-31⁶³ were found to be useful biocatalysts for the enantioselective desymmetrization of prochiral ketones, both giving similar results as CHMO from *Acinetobacter*. A BVMO from *Xanthobacter* sp. ZL5 was reported to be capable of the production of enantiomerically pure lactones from structurally demanding substrates **109** and **111–114**.¹⁹⁹ Whole cells expressing HAPMO oxidized several prochiral cyclobutanones to antipodal γ -butyrolactones when compared with CHMO_{Acineto}-mediated reactions, but in moderate yield and enantioselectivities.²⁰⁰ Interestingly, PAMO from *Thermobifida fusca* was used for the oxidation of 3-phenylpenta-2,4-dione (**107**) to give (R)-1-acetoxy-phenylacetone in 82% enantioselectivity, being the only BVMO-mediated desymmetrization of an aryl-aliphatic ketone to date.²⁰¹

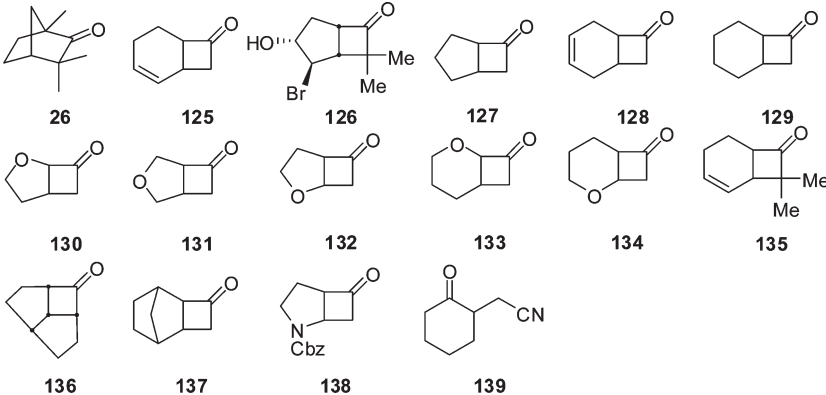
The screening efforts reported in this section were mainly focused on the identification of biocatalysts, which produce antipodal forms of the produced lactones, and as can be seen by the results for many substrate classes, this task could be achieved. An alternative strategy to gain access to enantioselective biocatalysts is via protein engineering techniques. Achievements and limitations of altering available biocatalysts themselves with respect to

desymmetrizations of prochiral ketones will be discussed in detail in section 7 of this review.

4.5.5. Regiodivergent and Regioselective Oxidations.

Probably the most remarkable BVMO-mediated transformation is the regiodivergent oxidation of ketones. In contrast to most traditional peracid oxidations, BVMO-catalyzed oxidations yield two regioisomeric products for selected substrates. The expected “normal” lactone is produced by the migration of the more nucleophilic center, in most cases the higher substituted carbon atom adjacent to the ketone functionality, whereas a second product, the “abnormal” lactone, is formed by migration of the less nucleophilic carbon center. In many cases, the two regioisomeric lactones are obtained in a ratio of 1:1 in high enantioselectivities. These results can be explained by the fact that each antipodal substrate is positioned differently in the active site of the enzyme and therefore different groups are arranged antiperiplanar to the peroxide functionality in the Criegee intermediate. Since the BV reaction occurs under stereoelectronic control, this restricted conformation of the transition state dictates which group migrates in the BV oxidation, regardless of the nucleophilicity of the carbon atom, therefore differing considerably from the chemical BV oxidation where the more substituted carbon center migrates preferentially.²⁰²

The first regiodivergent transformation using BVMOs was observed during the oxidation of racemic fenchone (**26**), which gave a mixture of 1,2- and 2,3-fencholide in a ratio of 9:1 with cells of *Corynebacterium* sp. in 1965. In comparison, the chemical oxidation of fenchone yielded the two corresponding lactones in a ratio of 4:6.¹⁰⁵ The first enantioselective regiodivergent BVMO-mediated oxidation was observed by Alphand and Furstoss nearly 25 years later.²⁰³ The bioconversion of bicyclo[3.2.0]hept-2-en-6-one (**122a**) with whole cells of *Acinetobacter* TD63 yielded the “normal” as well as the “abnormal” lactone in a ratio of 1:1 in a highly regio- and enantiospecific reaction in good yield (Figure 31). The expected “normal” lactone **123a** was used as an intermediate for the synthesis of the Corey lactone, a versatile building block in the synthesis of prostaglandins. *C. echinulata* was identified by the same group as an extremely useful



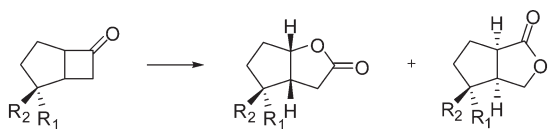
Substrate	Biocatalyst	Yield (%)	Ratio	% ee "normal"	% ee "abnormal"	Reference
26	<i>Corynebacterium</i> sp.	n. r.	90:10	n. r.	n. r.	105
125	<i>C. destructans</i>	32	100:0	27 (1 <i>R</i> ,6 <i>S</i>)	n. r.	208a
	<i>Acinetobacter</i> TD63	58	40:39	>95 (1 <i>R</i> ,6 <i>S</i>)	>95 (1 <i>S</i> ,6 <i>R</i>)	205
126	<i>Acinetobacter</i> NCIMB 9871	50 conv	100:0	(-)-31	n. d.	209a
	<i>P. putida</i> AS1	11	0:100	n. d.	92	209d
127	<i>Acinetobacter</i> NCIMB 9871	39	36:31	>95 (1 <i>S</i> ,5 <i>S</i>)	>95 (1 <i>R</i> ,5 <i>S</i>)	205
	CHMO _{Brev1} - <i>E. coli</i>	78	1:1	>99 (1 <i>S</i> ,5 <i>S</i>)	>99 (1 <i>R</i> ,5 <i>S</i>)	212a
	CHMO _{Brev2} - <i>E. coli</i>	78	55:45	76 (1 <i>S</i> ,5 <i>S</i>)	91 (1 <i>R</i> ,5 <i>S</i>)	212a
128	<i>Acinetobacter</i> TD63	46	43:41	92 (1 <i>S</i> ,6 <i>S</i>)	>95 (1 <i>S</i> ,6 <i>R</i>)	205
129	<i>Acinetobacter</i> TD63	36	47:30	>95 (1 <i>S</i> ,6 <i>S</i>)	53 (1 <i>S</i> ,6 <i>R</i>)	205
	CHMO _{Brev1} - <i>E. coli</i>	83	60:40	78 (1 <i>S</i> ,6 <i>S</i>)	>99 (1 <i>S</i> ,6 <i>R</i>)	212a
	CHMO _{Brev2} - <i>E. coli</i>	79	96:4	2 (1 <i>S</i> ,5 <i>S</i>)	>99 (1 <i>S</i> ,6 <i>R</i>)	212a
130	<i>Acinetobacter</i> NCIMB 9871	67	35:32	90 (1 <i>R</i> ,5 <i>S</i>)	>98 (1 <i>R</i> ,5 <i>S</i>)	206
131	<i>Acinetobacter</i> NCIMB 9871	70	1:1	97 (1 <i>R</i> ,5 <i>R</i>)	>98 (1 <i>S</i> ,5 <i>R</i>)	206
	CHMO _{Brev1} - <i>E. coli</i>	74	65:35	99 (1 <i>R</i> ,5 <i>R</i>)	>99 (1 <i>S</i> ,5 <i>R</i>)	212a
	CHMO _{Brev2} - <i>E. coli</i>	79	98:2	6 (1 <i>R</i> ,5 <i>R</i>)	84 (1 <i>S</i> ,5 <i>R</i>)	212a
132	<i>Acinetobacter</i> NCIMB 9871	74	33:41	>98 (1 <i>S</i> ,5 <i>S</i>)	>98 (1 <i>R</i> ,5 <i>S</i>)	206
	CHMO _{Brev1} - <i>E. coli</i>	72	46:54	97 (1 <i>S</i> ,5 <i>S</i>)	>99 (1 <i>R</i> ,5 <i>S</i>)	212a
	CHMO _{Brev2} - <i>E. coli</i>	83	62:38	57 (1 <i>S</i> ,5 <i>S</i>)	97 (1 <i>R</i> ,5 <i>S</i>)	212a
133	<i>Acinetobacter</i> NCIMB 9871	66	1:1	70 (1 <i>R</i> ,6 <i>S</i>)	>98 (1 <i>R</i> ,6 <i>S</i>)	206
134	<i>Acinetobacter</i> NCIMB 9871	78	60:18	33 (1 <i>S</i> ,6 <i>S</i>)	>98 (1 <i>S</i> ,6 <i>R</i>)	206
	CHMO _{Brev1} - <i>E. coli</i>	64	75:25	97 (1 <i>S</i> ,6 <i>S</i>)	>99 (1 <i>S</i> ,6 <i>R</i>)	212a
	CHMO _{Brev2} - <i>E. coli</i>	71	96:4	0 (1 <i>S</i> ,6 <i>S</i>)	>99 (1 <i>S</i> ,6 <i>R</i>)	212a
	BVMO _{Myco} - <i>E. coli</i>	50 conv	0:100	n. a.	>99 (1 <i>S</i> ,6 <i>R</i>)	47
135	<i>P. putida</i> AS1	60	11:59	n. d.	59	209d
136	CHMO _{Acineto} - purified	n. a.			>98	202b
	CPMO - purified	n. a.			>98	202b
	2,5-DKCMO - purified	n. a.			> 98 (-)	202b
	3,6-DKCMO - purified	n. a.			> 98 (-)	202b
137	CHMO _{Brev1} - <i>E. coli</i>	77	52:48	89	>99	212a
	CHMO _{Brev2} - <i>E. coli</i>	86	69:31	44	>99	212a
138	CHMO _{Acineto} - <i>E. coli</i>	48	3:4:1	96 (1 <i>R</i> ,5 <i>R</i>)	93 (1 <i>R</i> ,5 <i>S</i>)	214
	<i>Acinetobacter</i> TD63	40	1:2.6	94 (1 <i>R</i> ,5 <i>R</i>)	87 (1 <i>R</i> ,5 <i>S</i>)	214
139	CHMO _{Acineto} - <i>E. coli</i>	68	32:36	>99 (i <i>R</i>)	>99 (i <i>S</i>)	163

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation or absolute configuration of product is given in parentheses; ^c n. a. = not applicable; ^d n. r. = not reported; ^e n. d. = not determined.

Figure 32. BVMO-mediated regiodivergent oxidations.

biocatalyst since it only produces the “abnormal lactone” **124a** in a kinetic resolution process and therefore the isolation step was simplified. The “abnormal” lactone was used as a starting material for the chemoenzymatic total synthesis of various brown algae pheromones (multifidene (**146**), viridene (**147**), and caudoxirene (**148**)) and (–)-sarkomycin (**143**) in subsequent publications.²⁰⁴ These initial results prompted various research groups to investigate the oxidation of fused bicyclic ketones, bearing a cyclobutanone structural motif with fungal cells, bacterial cells, or enzyme preparations. Several bicyclic [*n*.2.0] ketones (**127**–**129**, Figure 32)²⁰⁵ as well as structurally related compounds

with an additional oxygen atom in the substrate (**130**–**134**, Figure 32)²⁰⁶ were subjected to bacterial whole cell fermentations with *Acinetobacter* TD63 and *Acinetobacter* NCIMB 9871 by the group of Furstoss. For all substrates, high regiodivergent and enantioselective oxidations were observed. Petit and Furstoss further showed that the normal lactone derived from 2-oxobicyclo[3.2.0]heptan-7-one (**130**) could serve as a suitable building block for the synthesis of clerodane derivatives.²⁰⁷ In addition, bicyclo[3.2.0]hept-2-en-6-one (**122a**) has been used extensively as a model substrate for the screening of microbial strains in order to detect new BVMO activities.^{182b,208}



Substrate	R ₁	R ₂	Biocatalyst	Yield (%)	Ratio 141 : 142	% ee "normal" ^a	% ee "abnormal" ^a	Reference
140a	H	Br	<i>Acinetobacter</i> NCIMB 9871	93	66:34	48 (1 <i>R</i> ,5 <i>R</i>)	97 (1 <i>R</i> ,5 <i>S</i>)	211
140b	Br	H	<i>Acinetobacter</i> NCIMB 9871	79	62:38	54 (1 <i>R</i> ,5 <i>R</i>)	95 (1 <i>R</i> ,5 <i>S</i>)	211
140c	OBn	H	<i>Acinetobacter</i> NCIMB 9871	71	54:46	44 (1 <i>R</i> ,5 <i>R</i>)	78 (1 <i>R</i> ,5 <i>S</i>)	211
140d	H	OBn	<i>Acinetobacter</i> NCIMB 9871	78	32:68	rac	20 (1 <i>R</i> ,5 <i>S</i>)	211
140e		=O	<i>Acinetobacter</i> NCIMB 9871	36	79:21	92 (1 <i>R</i> ,5 <i>R</i>)	97 (1 <i>R</i> ,5 <i>S</i>)	211
140f	OH	H	<i>Acinetobacter</i> NCIMB 9871	75	52:48	90 (1 <i>R</i> ,5 <i>R</i>)	>98 (1 <i>R</i> ,5 <i>S</i>)	211

^a absolute configuration of product is given in parentheses.

Figure 33. Regiodivergent oxidations of 2-substituted bicyclo[3.2.0]heptan-6-one derivatives.

The oxidation of substituted [3.2.0]heptan-6-one derivatives such as compound **126** by BVMOs from either *Acinetobacter* NCIMB 9871 or *P. putida* NCIMB 10007 were extensively studied by the groups of Willets and others.^{156,209} Interestingly the 7-exo-7-endophenylbicyclo derivative underwent allylic oxidation in addition to the BV oxidation when subjected to whole cell fermentations with *C. echinulata* NRRL 3655.²¹⁰ The regio- and enantioselective oxidation of some 2-substituted bicyclo[3.2.0]heptan-6-one derivatives (**140a–f**) led to a chemoenzymatic synthesis of sarkomycin A (**143**) by Königsberger and Griengl (Figure 33, Figure 34).²¹¹ *meso*-Tricyclo[4.2.1.0^{3,9}]-nonan-2-one (**136**) was prepared by Kelly and co-workers as a probe for regiodivergent transformations.²⁰² Both whole cells and crude and purified enzyme extracts of type 1 (including CHMO_{Acineto}, CPMO, MO2) and type 2 BVMOs (2,5- and 3,6-DKCMO) were used as biocatalysts, and in every case, the tricyclic ketone was transformed with >96% ee. Interestingly, type I BVMOs gave uniformly one enantiomer and type 2 BVMOs the other.²⁰² Different stereoselectivities for the regiodivergent oxidation of fused bicyclic ketones by recombinant whole cells expressing various bacterial BVMOs were observed by the group of Mihovilovic.²¹² Whole cells expressing BVMOs belonging to the previously classified CHMO family gave regiodivergent oxidations to “normal” and “abnormal” lactones in high optical purities, whereas racemic “normal” lactones were produced by BVMOs from the CPMO family.²¹² The complementary behavior of the two classes of enzymes is in agreement with the previous results obtained in desymmetrization reactions.⁷⁵ Whole cells expressing a BVMO from *Xanthobacter* sp. Z15 were found to cluster with BVMOs from the CHMO family in their biocatalytic performance toward racemic bicyclic ketones⁶⁸ and a BVMO from *Mycobacterium tuberculosis* H37Rv was found to resolve the same class of substrates by oxidizing only one enantiomer to preferentially one type of regioisomeric lactone (Figure 34).⁴⁷ For example, 2-oxabicyclo[4.2.0]octan-7-one (**134**, Figure 32) was oxidized exclusively to the abnormal lactone in a kinetic resolution process. Both substrate and lactone were recovered in excellent yield and identified as intermediates in the synthesis of prostanoid derivatives.⁴⁷

Besides the oxidation of fused bicyclic ketones, the regiodivergent BVMO-mediated oxidation of 3-substituted cyclopentanones (Figure 35) and 3-substituted cyclohexanones (Figure 36) have been studied in depth by the groups of Kayser and Stewart.^{158,160b,213} The outcome of such transformations are less predictable for both chemical and enzymatic transformations, since both carbon atoms adjacent to the ketone functionality

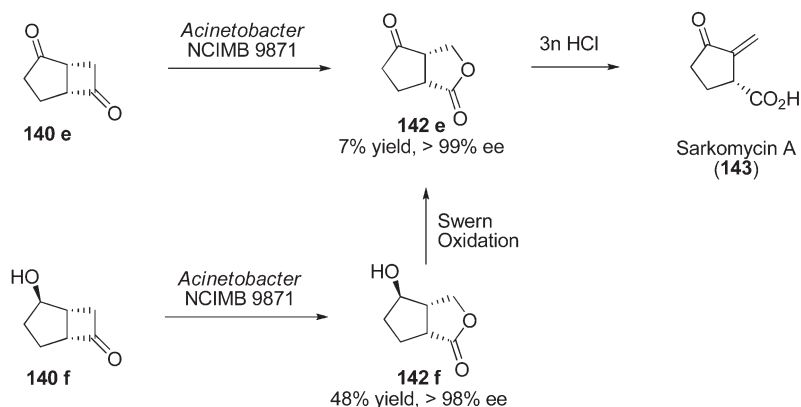
possess nearly identical nucleophilicity and therefore such transformations of racemic ketones can give four products. Initial studies were conducted with engineered yeast cells overexpressing CHMO from *Acinetobacter* NCIMB 9871 and enantioselective regiodivergent behavior was reported for the conversion of 3-methyl- and 3-ethyl-substituted cyclohexanones (**150a** and **150b**), but in low *E* values in favor of the *R*-ketone.^{160b} Cyclohexanones with sterically more demanding substituents (**150c–f**) were oxidized regioselectively to the proximal lactones in moderate *E* values.^{160b} In contrast, CPMO expressed in *E. coli* displayed high proximal regioselectivity for both 3-substituted cyclohexanones and 3-substituted cyclopentanones.²¹³ Optically pure 4- and 5-substituted lactones were prepared successfully by combining an asymmetric copper-catalyzed reduction step of α,β -unsaturated ketone derivatives followed by the regioselective oxidation with whole cells expressing CPMO.²¹³ In a subsequent study by Stewart, nine BVMOs recombinantly expressed in *E. coli* were screened for their synthetic utility as regio- and enantioselective biocatalysts for the production of 4- and 5-substituted lactones.¹⁶² In some cases, for *n*-Pr and *n*-Bu substituents, both regio- and enantioselective BV oxidations were observed, but more commonly only one type of selectivity was displayed by the biocatalyst applied.¹⁶²

The formation of “normal” and “abnormal” lactones was also observed during the oxidation of terpenones (Figure 37).¹¹⁴ A small library of BVMOs expressed in *E. coli*, including members of the CHMO and CPMO family, was applied to the oxidation of optically pure terpenones. CHMO-type enzymes gave regiodivergent oxidations to both “normal” and “abnormal” lactones depending on the absolute configuration of the substrate. CPMO-type BVMO-mediated oxidations gave more sluggish reactions with predominantly “abnormal” lactones as products as shown in Figure 37. (–)-Menthone (**39**) was not converted by any of the tested BVMOs.¹¹⁵

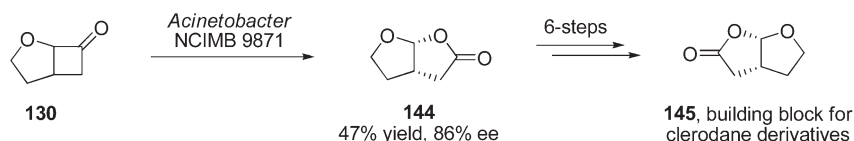
Other enantioselective regiodivergent BVMO-mediated oxidations have been reported for the oxidation of aliphatic ketones,¹⁷¹ the *N*-protected Geisman–Waiss lactone (**138**),²¹⁴ and 2-(cyanomethyl)cyclohexanone (**139**)¹⁶³ with recombinant whole cells expressing CHMO from either *Acinetobacter* NCIMB 9871 or the native strain *Acinetobacter* TD63.

The oxidation of norcamphor derivatives takes a unique position in BVMO-mediated transformations, since norbornanones were used as substrates in regioselective oxidations, kinetic resolutions, and BVMO-mediated regiodivergent transformations (Figure 38). Whole cell mediated oxidations with either *Comamonas* NCIMB 9872 or *Acinetobacter* NCIMB 9871 were reported to give higher regioselectivities in the oxidation of

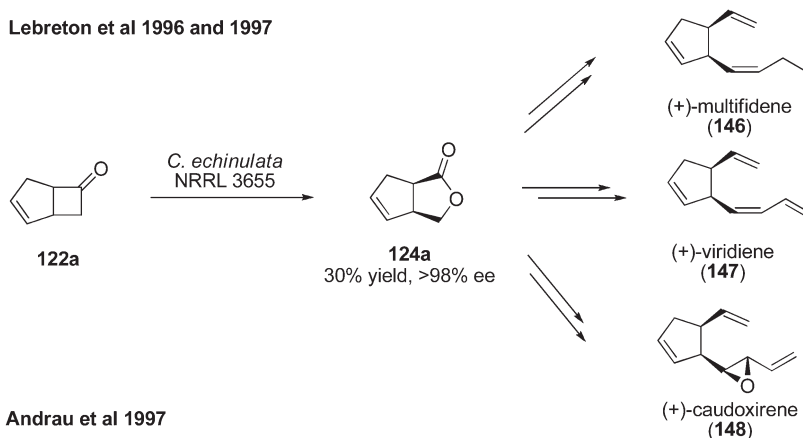
Königsberger and Griengl 1994



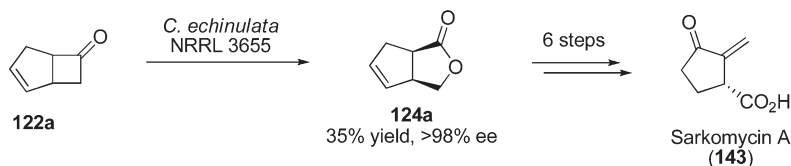
Petit and Furstoss 1995



Lebreton et al 1996 and 1997



Andrau et al 1997



Snajdrova et al 2006

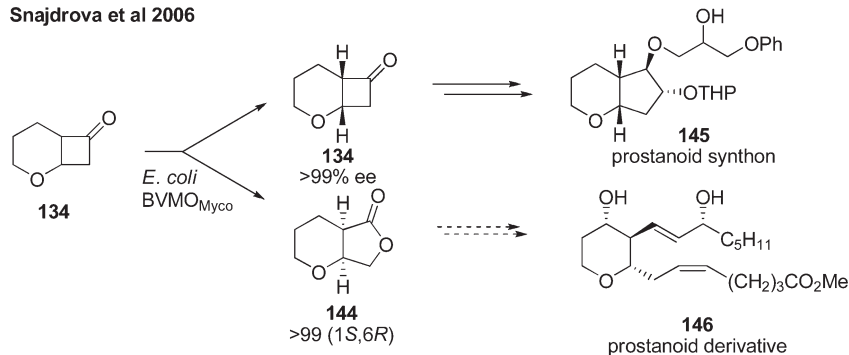


Figure 34. Applications of chiral synthons derived from BVMO-mediated regiodivergent reactions.

norcamphor and 5,7-disubstituted norcamphor derivatives than the equivalent peracid-catalyzed reactions.²¹⁵ The chemoenzymatic synthesis of 6'-fluorocarboxylic nucleosides such as **156**

and the enantioselective preparation of a potential precursor of azadirachtin **157**, a potent antifeedant and growth regulator, was reported by Willets and co-workers (Figure 39).²¹⁶ In both

147 a-j → **148 a-j** ("proximal" lactone) + **149 a-j** ("distal" lactone)

Substrate	R	Biocatalyst ^a	Yield (%)	Ratio 148 : 149	% ee "proximal" ^b	% ee "distal" ^b	Reference
147a	Me	<i>Comamonas</i> NCIMB 9872	70 conv	59:4	rac	rac	158
		CHMO _{Acineto} - yeast	95	13:87	9	36	160c
		CPMO - <i>E. coli</i>	68	100:0	rac	n. a.	213
147b	(R)-Me	CPMO - <i>E. coli</i>	62	100:0	> 99 (R)	n. a.	213
		CHMO _{Acineto} - yeast	80	80:20	33	19	160c
		CPMO - <i>E. coli</i>	78	100:0	rac	n. a.	213
147c	(R)-Et (86% ee)	CPMO - <i>E. coli</i>	69	100:0	98 (R)	n. a.	213
		CHMO _{Acineto} - yeast	44	83:17	33	66	160c
		CPMO - <i>E. coli</i>	70	100:0	rac	n. a.	213
147d	(S)-iso-Pr (87% ee)	CHMO _{Acineto} - <i>E. coli</i>	58	33:67	97 (R)	93 (S)	213
		CPMO - <i>E. coli</i>	75	100:0	86 (R)	n. a.	213
147e	allyl	CHMO _{Acineto} - yeast	42	44:56	23	n. d.	160c
147f	<i>n</i> -Bu	CHMO _{Acineto} - yeast	34	99:1	38	n. a.	160c
		CPMO - <i>E. coli</i>	76	100:0	rac	n. a.	213
	(S)- <i>n</i> -Bu (90% ee)	CHMO _{Acineto} - <i>E. coli</i>	88	95:5	90 (S)	n. a.	213
		CPMO - <i>E. coli</i>	84	100:0	92 (S)	n. a.	213
	<i>n</i> -Hex	CHMO _{Acineto} - yeast	20	99:1	60	n. a.	160c
147h	<i>n</i> -Oct	CHMO _{Acineto} - yeast	19	99:1	16	n. a.	160c
147i	<i>n</i> -Undec	CHMO _{Acineto} - yeast	20	99:1	rac	n. a.	160c
147j	(S)- (CH ₂) ₅ Ph (88% ee)	CHMO _{Acineto} - <i>E. coli</i>	80	100:0	99 (S)	n. a.	213
		CPMO - <i>E. coli</i>	80	100:0	98 (S)	n. a.	213

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b absolute configuration of product is given in parentheses; ^c n. a. = not applicable; ^d n. d. = not determined.

Figure 35. BVMO-mediated oxidations of 3-substituted cyclopentanones.

150 a-f → **151 a-f** ("proximal" lactone) + **152 a-f** ("distal" lactone)

Substrate	R	Biocatalyst ^a	Yield (%)	Ratio 150 : 151	<i>E</i> - value	% ee "proximal" ^b	% ee "distal" ^b	Reference
150a	Me	CHMO _{Acineto} - yeast	n. r.	n. r.	2	>98 (-)	>98 (-)	160b
		CHMO _{Acineto} - <i>E. coli</i>	77	100:0	n. a.	>99 (R)	n. a.	213
		CPMO - <i>E. coli</i>	75	100:0	n. a.	>99 (R)	n. a.	213
		CHMO _{Acineto} - <i>E. coli</i>	60	0:100	n. a.	n. a.	99 (S)	213
		CPMO - <i>E. coli</i>			no reaction			213
150b	Et	CHMO _{Acineto} - yeast	n. r.	n. r.	18	70 (-)	70 (-)	160b
		CHMO _{Acineto} - <i>E. coli</i>	89	94:6	n. a.	94 (R)	>99 (S)	213
		CPMO - <i>E. coli</i>	87	100:0	n. a.	80 (R)	n. a.	213
150c	<i>n</i> -Pr	CHMO _{Acineto} - yeast	80	96:2	51	>98 (-)	n. r.	160b
150d	<i>i</i> -Pr	CHMO _{Acineto} - yeast			no reaction			160b
150e	allyl	CHMO _{Acineto} - yeast	86	93:7	67	97 (-)	n. r.	160b
150f	<i>n</i> -Bu	CHMO _{Acineto} - yeast	77	98:2	8	56 (-)	n. r.	160b
		CHMO _{Acineto} - <i>E. coli</i>	38 (conv)	100:0	n. a.	56 (S)	n. a.	213
		CPMO - <i>E. coli</i>	85	100:0	n. a.	83 (S)	n. a.	213

^a *E. coli* or yeast indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation or absolute configuration of product is given in parentheses; ^c n. a. = not applicable; ^d n. r. = not reported.

Figure 36. BVMO-mediated oxidations of 3-substituted cyclohexanones.

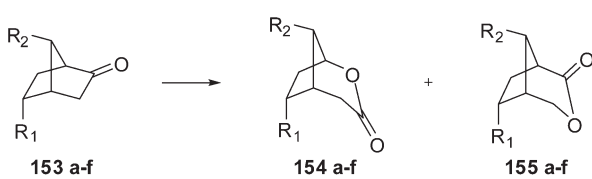
reaction sequences, the key step represented the kinetic resolution of a substituted norcamphor derivative. 5-Bromo-7-fluoro-norbornanone (**153c**) was resolved by whole cells of

Acinetobacter NCIMB 9871 and the optically pure remaining ketone (30% yield, >95% ee) was employed in the synthesis of the antiviral carbanucleoside **156**.^{216a} For the synthesis of the

Substrate	Biocatalyst ^a	Yield (%) "normal"	Yield (%) "abnormal"	Reference
(+)-trans-dihydrocarvone (36)	<i>Acinetobacter</i> NCIMB 9871	-	73	114
	CHMO _{Brev1} - <i>E. coli</i>	34	28	115
(-)-trans-dihydrocarvone (37)	<i>Acinetobacter</i> NCIMB 9871	80	-	114
	CPMO - <i>E. coli</i>	-	18	115
(+)-menthone (38)	<i>Acinetobacter</i> NCIMB 9871	90	-	114
(-)-menthone (39)	<i>Acinetobacter</i> NCIMB 9871	no reaction		114
(+)-cis-dihydrocarvone (40)	CHMO _{Brev1} - <i>E. coli</i>	60	-	115
	CHMO _{Brachy} - <i>E. coli</i>	-	7	115
(-)-cis-dihydrocarvone (41)	CHMO _{Acineto} - <i>E. coli</i>	77	-	115
	CPMO - <i>E. coli</i>	-	28	115
(+)-carvomenthone (42)	CHMO _{Brev1} - <i>E. coli</i>	34	44	115
	CHMO _{Brachy} - <i>E. coli</i>	-	76	115
(-)-carvomenthone (43)	CHMO _{Brev1} - <i>E. coli</i>	71	-	115
	CPMO - <i>E. coli</i>	-	60	115

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst.

Figure 37. Regiodivergent oxidations of terpenes.



Substrate	R ₁	R ₂	Biocatalyst ^a	Yield (%)	Ratio 154 : 155	% ee "normal" ^b	% ee "abnormal" ^b	Reference
153a	H	H	<i>Comamonas</i> NCIMB 9872	100 conv	38:1	n. r.	n. r.	215a
			CHMO _{Acineto} - <i>E. coli</i>	91	97:3	12	88	212b
			CPMO - <i>E. coli</i>	85	75:25	33	56	212b
153b	OAc	F	<i>Acinetobacter</i> NCIMB 9871	44	100:0	n. r.	n. r.	215b
153c	Br	F	<i>Acinetobacter</i> NCIMB 9871	36	100:0	>95 (-)	n. r.	216a
153d	OH	H	MO1	11	100:0	>95 (1 <i>S</i> ,5 <i>S</i>)	n. r.	216b
153e	OAc	H	MO1	35	100:0	>95 (1 <i>S</i> ,5 <i>S</i>)	n. r.	216b
153f	OBn	H	MO1	39	100:0	>95 (1 <i>S</i> ,5 <i>S</i>)	n. r.	216b
			MO2	50	100:0	87 (1 <i>S</i> ,5 <i>S</i>)	n. r.	216b

^a *E. coli* indicates experiments with recombinant whole cell biocatalyst; ^b sign of specific rotation or absolute configuration of product is given in parentheses; ^c n. r. = not reported.

Figure 38. BVMO-mediated oxidations of norcamphor derivatives.

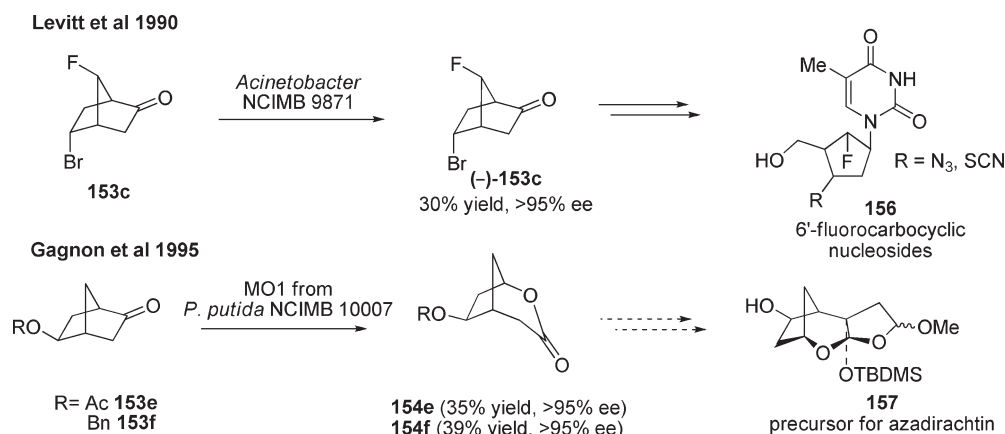


Figure 39. Applications of chiral synthons derived from BVMO-mediated oxidations of norcamphor derivatives.

intermediate of azadirachtin **157**, MOs from *P. putida* NCIMB 10007, especially MO1 (a mixture of 2,5-DKCMO and 3,6-DKCMO), were successfully applied to the resolution of

5-substituted bicyclo[2.2.1]heptan-2-ones **153e** and **153f** to give the desired "normal" lactone (39%, >95% ee).^{216b} An enantioselective oxidation of an unsaturated substituted norcamphor

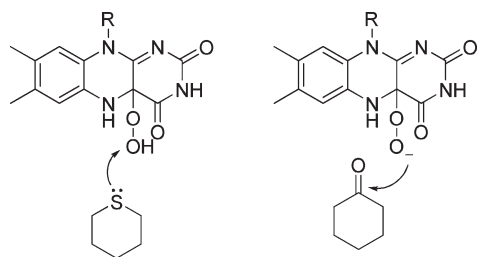


Figure 40. Ambivalent reactivity of 4 α -hydroperoxyflavin species illustrated by the examples of BV and sulfide oxidations.

derivative was reported by the group of Griengl and Furstoss, and the potential of the rearranged product as chiral synthon for the synthesis of prostaglandins was stated.²¹⁷ A study by Mihovilovic and co-workers showed that CHMO-type BVMOs generally give the normal lactones of norcamphor in low enantioselectivities; in contrast CPMO-type enzymes were reported to produce a 3:1 mixture of regioisomeric lactones with moderate enantioselectivities.^{212b}

In addition regiocomplementary lactones from benzo-fused ketones were obtained by the group of Gotor depending on the biocatalyst used. For example, HAPMO yielded exclusively the expected lactone 3,4-dihydrocoumarin from 1-indanone while a mutant of PAMO gave 1-isochromanone.²¹⁸

4.6. Baeyer–Villiger Monooxygenase-Mediated Heteroatom Oxidations

The first research group to investigate the action of BVMOs on the oxidations of heteroatom-containing xenobiotics was that of Walsh in 1982.²¹⁹ Purified CHMO from *Acinetobacter* transformed 4-tolyl ethyl sulfide (**158a**) to the corresponding (*S*)-sulfoxide **159a** in modest enantioselectivity. Mechanistic studies indicated that the sulfur atom acts as a nucleophile and attacks the terminal oxygen of the 4 α -hydroperoxyflavin species, differing considerably from the oxidation of ketones, where the hydroperoxide intermediate acts as the nucleophile, as shown in Figure 40.²²⁰ CHMO showed excellent activities toward the oxidation of sulfide substrates, processing tetrahydrothiopyran with 88% of the maximal velocity (V_{\max}) of cyclohexanone. Additionally high concentrations of sulfoxides were shown to be converted to the corresponding sulfones by CHMO, and other substrates, selenides, phosphates, boronic acids, and iodide, were identified.²²¹ Analogous to the CHMO-mediated oxidation of ketones, conversion of a chiral boronic acid was shown to occur with retention of configuration at the migrating carbon center.²²² Initial attempts to generate mechanism-based inactivators by enzyme-mediated oxidation of allyl sulfides and selenides failed.²²³ Subsequent research on the oxidation of cyclic thiol ester substrates by Walsh and co-workers showed that the products derived from 2-thiacyclohexanone and thiocarbonates act as suicide inhibitors.²²⁴ In contrast, the 3- and 4-thiacyclohexanone derivatives were converted exclusively to the lactones with no detectable sulfoxides formed, reflecting the preferential activity of CHMO from *Acinetobacter* toward the oxidation of the carbonyl compared with the oxidation of sulfur.²²⁴ Two reviews on the pioneering work by Walsh and co-workers on BVMO-mediated oxidations of heteroatoms were composed in the late 1980s.^{10i,225}

Based on these results and the upcoming need for chiral sulfoxides as valuable reagents for the synthesis of natural products,

as sulfur ligands, and as stereodirecting groups on preparative scale,²²⁶ the group of Carrea tested partially purified enzyme preparations of CHMO from *Acinetobacter* in the biooxidation of numerous alkyl aryl sulfides, substituted alkyl aryl sulfides, dialkyl sulfides, and dialkyl disulfides (Figures 41 and 42).²²⁷ The stereoselectivity of the CHMO_{*Acineto*}-mediated oxidation was found to be highly dependent on the structure of the sulfide starting material, and products with both (*R*)- and (*S*)-configuration were obtained. For example, (*R*)-methyl phenyl sulfoxide (**159b**) was isolated in 99% ee, whereas oxidation of the structurally closely related ethyl 4-fluorophenyl sulfide (**158h**) gave the corresponding (*S*)-sulfoxide **159h** in 93% ee. Similar results were obtained for the oxidation of alkyl aryl sulfides substituted with functional groups²²⁸ and benzyl alkyl sulfide derivatives.²²⁹ Enantioselectivities for the oxidation of disulfides with CHMO_{*Acineto*} were found to be rather poor, except for the transformation of *tert*-butyl disulfide (**160a**), which yielded optically pure (*R*)-*tert*-butyl-butanethiosulfonate (**161a**).^{227,230} Predominantly (*R*)-selective oxidations were reported for dialkyl sulfides (**158o–q**) as substrates with CHMO_{*Acineto*} to give the corresponding dialkylsulfoxides in moderate to excellent optical purities.²³¹ Based on earlier published concepts¹¹⁴ and the results obtained from the oxidations of sulfides, an active site model of CHMO from *Acinetobacter* was proposed by the group of Ottolina in order to predict the stereoselectivity of CHMO_{*Acineto*}-mediated sulfide oxidations and biooxidations of ketones.²³²

In addition to the work on the oxidation of monosulfide-containing xenobiotics, the enzymatic oxidation of dithioacetals (Figure 43) has been investigated in detail, since the corresponding 1,3-dithioacetal monosulfoxides can serve as chiral acyl anion equivalents.^{10g} Initial reports by Colonna and co-workers demonstrated that the CHMO_{*Acineto*}-mediated conversion of three 1,3-dithioacetals, **162a–c**, yielded enantiomerically pure (*R*)-monosulfoxides in good yield besides small amounts of monosulfones.²³³ The authors further showed that the excellent ee values were derived from both asymmetric oxidations of the starting materials and kinetic resolution of the monosulfoxides with favorable rates for oxidation of the (*S*)-enantiomers.²³³ The study was extended to the oxidation of 2-substituted thianes and dithiolanes, **162f–k**, in order to test the diastereo- and enantiotopic preference of CHMO_{*Acineto*}.²³⁴ Moderate to low enantioselectivities were reported for the enzymatic oxidation of *meso*-dialkyl derivatives (**162c–e**) and a correlation between the bulkiness of the substituents and a decrease in ee values of the corresponding monosulfoxides was established. Exclusive or preferential formation of the *trans*-monosulfoxides derived from 2-substituted dithianes and dithiolanes **162f–k** was reported. CHMO oxidized 2-methyl- and 2-benzoyl-1,3-dithiane (**162f**, **162h**) in good to excellent enantioselectivities, whereas others substrates gave significantly lower ee values.²³⁴ Moreover enzymatic oxidations of dithioacetals and dithioketals on preparative scale were reported by the group of Furstoss using native whole cells of *Acinetobacter* NCIMB 9871, *Acinetobacter* TD63, *Comamonas* NCIMB 9872, and *C. echinulata* NRRL 3655 as biocatalysts. The results were compared with experiments with purified enzyme extracts of *Acinetobacter* NCIMB 9871.²³⁵ Bacterial strains were found to give higher yields of monosulfoxides than experiments with *C. echinulata*, presumably due to the degradation of products by the fungal strain. Results for either whole cells of *Acinetobacter* NCIMB 9871 or purified CHMO from this strain were very similar, and for some substrates, whole cells of *Comamonas* NCIMB 9872 displayed an enantiodivergent

Substrate	R ₁	R ₂	Biocatalyst	Yield or Conversion (%)	% ee ^a	Reference
158a	<i>p</i> -Me-Ph	Et	CHMO _{Acineto}	n. r.	73 (S)	219
			CHMO _{Acineto}	89	89 (S)	227
			<i>Comamonas</i> NCIMB 9872	94	100 (S)	238
158b	Ph	Me	CHMO _{Acineto}	88	99 (R)	227
			<i>Comamonas</i> NCIMB 9872	95	84 (S)	238
			<i>X. autotrophicus</i> DSM 431	75	100 (R)	238
			PAMO	94	44 (R)	201
			HAPMO	96	99 (S)	241
158c	<i>p</i> -Me-Ph	Me	CHMO _{Acineto}	94	37 (S)	227
			<i>P. putida</i> NCIMB 10007	98	64 (S)	237
			<i>Comamonas</i> NCIMB 9872	95	84 (S)	238
			2,5-DKCMO	20	75 (S)	209g
158d	<i>p</i> -F-Ph	Me	3,6-DKCMO	30	40 (S)	209g
			CHMO _{Acineto}	91	92 (R)	227
			<i>Comamonas</i> NCIMB 9872	90	83 (S)	238
158e	Bn	Me	CHMO _{Acineto}	97	54 (R)	227
			PAMO	29	94 (S)	201
			HAPMO	55	85 (S)	241
158f	Ph	Et	CHMO _{Acineto}	86	47 (R)	227
			<i>Acinetobacter</i> NCIMB 9871	57	27 (R)	237
			HAPMO	86	99 (S)	241
158g	<i>p</i> -Me-Ph	Me	CHMO _{Acineto}	94	37 (S)	227
			<i>Acinetobacter</i> NCIMB 9871	94	26 (S)	237
			<i>P. putida</i> NCIMB 10007	98	64 (S)	237
			2,5-DKCMO	20	75 (S)	209g
			3,6-DKCMO	30	40 (S)	209g
158h	<i>p</i> -F-Ph	Et	PAMO	68	10 (R)	201
			CHMO _{Acineto}	93	93 (S)	227
			HAPMO	52	81 (S)	241
158i	Ph	CH ₂ -CN	CHMO _{Acineto}	90	92 (R)	228
158j	Ph	(CH ₂) ₂ -Cl	CHMO _{Acineto}	75	93 (S)	228
158k	Bn	Et	CHMO _{Acineto}	80	67 (S)	229
			2,5-DKCMO	20	2 (S)	239
			3,6-DKCMO	41	9 (R)	239
			PAMO	36	98 (S)	201
158l	Bn	<i>iso</i> -Bu	HAPMO	52	81 (S)	241
			CHMO _{Acineto}	90	90 (S)	229
			HAPMO	44	51 (R)	241
158m	Ph-(CH ₂) ₂	Me	CHMO _{Acineto}	95	40 (R)	229
158n	<i>p</i> -NH ₂ -Bn	Me	HAPMO	41	96 (S)	241
			CHMO _{Acineto}	100	65 (R)	232c
158o	cyclo-Pentl	Me	CHMO _{Acineto}	80	>98 (R)	231
158p	<i>tert</i> -Bu	Me	CHMO _{Acineto}	98	99 (R)	227
158q	Oct	Me	CHMO _{Acineto}	50	50 (S)	231
			2,5-DKCMO - purified	6	36 (S)	209g
			3,6-DKCMO - purified	20	16 (S)	209g

^a absolute configuration of product is given in parentheses; ^b n. r. = not reported.

Figure 41. Selected BVMO-catalyzed oxidations of sulfides.

trend to results with CHMO.²³⁵ A similar study was conducted by the groups of Kayser and Stewart who applied recombinant baker's yeast and *E. coli* expressing CHMO from *Acinetobacter* NCIMB 9871 to enantioselective oxidations of several sulfides, dithianes, and dithiolanes. In most cases, overoxidations of sulfoxides were observed using whole cell biocatalysts, but generally enantioselectivities and diastereoselectivities were identical to oxidations with purified CHMO (Figure 43).^{33a,236}

A few other BVMOs have been applied to enantioselective sulfide oxidations, as either whole cells, enzyme preparations, or

recombinant proteins. Whole cell mediated enantioselective transformations of aryl alkyl sulfides to sulfoxides have been reported with camphor-grown *P. putida* NCIMB 10007,²³⁷ *Pseudomonas* sp. NCIMB 9872, *X. autotrophicus* DSM 431 (NCIMB 10811), and the black yeast NV2.²³⁸ Interestingly whole cells of *Comamonas* NCIMB 9872 and *P. putida* NCIMB 10007 gave in some cases (e.g., **162a**) opposite enantioselectivity to results obtained with either whole cells or isolated CHMO from *Acinetobacter* NCIMB 9871. Data collected from enantioselective oxidations of 23 sulfides with enzyme preparations of

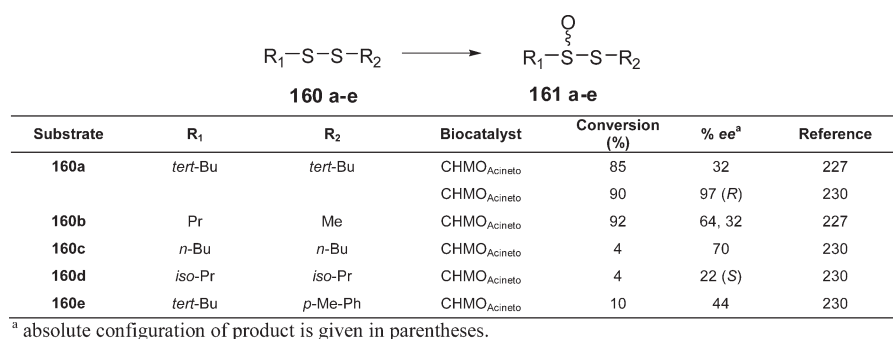


Figure 42. BVMO-catalyzed oxidations of disulfides.

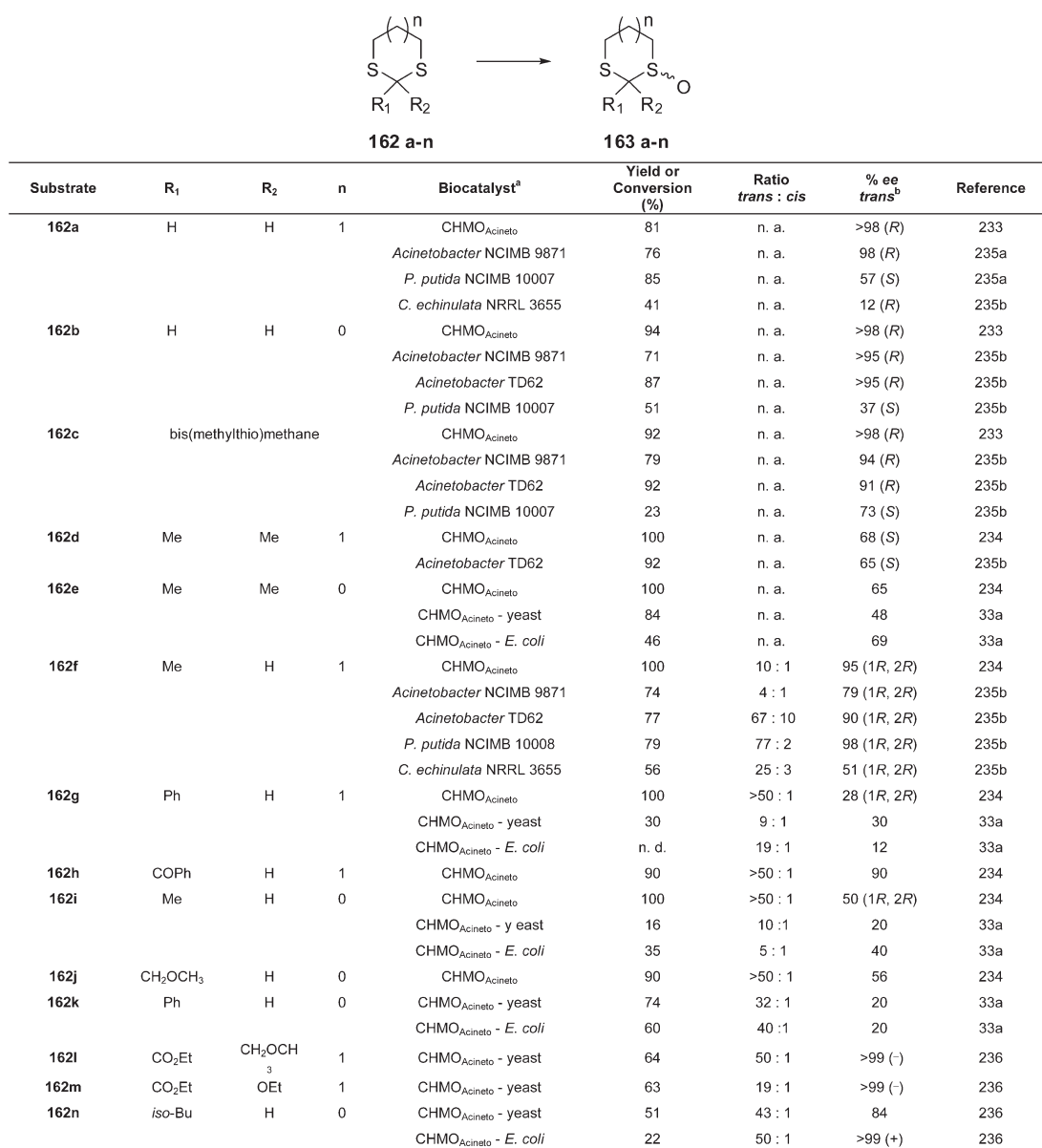


Figure 43. BVMO-mediated oxidations of dithioacetals.

2,5-DKCMO and 3,6-DKCMO from *P. putida* NCIMB 10007 were used to build active site models of both isozymes based on the same principles as Ottolina's model for CHMO_{Acinetobacter}.²³⁹ Recombinant PAMO from *T. fusca* proved to be an excellent biocatalyst for the enantioselective oxidation of methyl and ethyl benzyl sulfide (**158e**, **158k**) to the corresponding (*S*)-sulfoxides, whereas other sulfoxides produced from alkyl phenyl and alkyl benzyl sulfides were produced in low to moderate enantioselectivities.²⁰¹ In addition, PAMO resolved racemic ethyl benzyl sulfoxide with high enantioselectivity (*E* = 110), and thus this enzyme differs considerably from CHMO_{Acinetobacter}, which could not be exploited for kinetic resolution processes due to the slow oxidation rates of sulfoxides to sulfones. The effect of organic solvents on the biocatalytic properties of HAPMO, PAMO, and EtaA was investigated by de Gonzalo and co-workers.²⁴⁰ Interestingly the addition of methanol (30%) to PAMO-catalyzed oxidations altered significantly the observed enantioselectivities. The most striking example is the improvement of the optical purity of the product obtained from PAMO-mediated oxidation of ethyl phenyl sulfide, which improved from 33% (*S*) in buffer to 87% (*R*) in buffer and methanol.²⁴⁰ Probably the most successful BVMO for the enantioselective oxidation of sulfides to the corresponding sulfoxides is HAPMO from *P. fluorescens* ACB.²⁴¹ Biooxidation of a wide range of aryl and benzyl alkyl sulfides gave the corresponding sulfoxides in good to excellent enantioselectivities, and in contrast to other BVMOs, HAPMO displayed a clear trend for the production of the (*S*)-sulfoxides (Figure 41) and was shown to accept sulfoxides as substrates, but with unusable enantioselectivities.²⁴¹

Other synthetically interesting BVMO-mediated transformations of heteroatoms are the CHMO_{Acinetobacter} catalyzed oxidations of racemic cyclic sulfites to cyclic sulfates (Figure 44) and asymmetric oxidations of amines (Figure 45). Colonna and co-workers reported on the partial success in the kinetic resolution

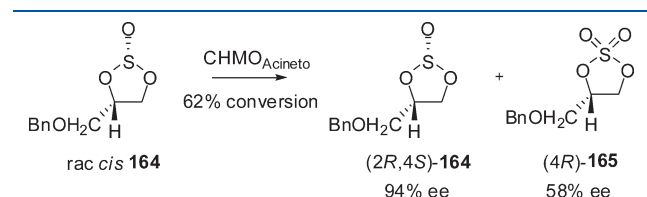


Figure 44. Enantioselective oxidation of sulfites to sulfates.

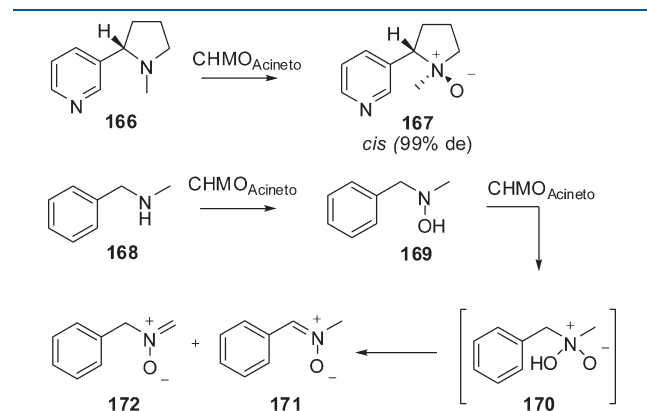


Figure 45. CHMO_{Acinetobacter}-mediated oxidation of amines.

of *cis*- and *trans*-4-benzyloxymethyl-1,3,2-dioxathiolane 2-oxide (**164**) as well as *cis*- and *trans*-methyl dioxathiolane 2-oxide derivatives. For example, at a conversion of 62%, the ee of the remaining *cis*-benzyloxymethyl derivative **164** was found to be 94% and the ee of the corresponding sulfate **165** was 58%.²⁴² Asymmetric oxidations of tertiary amines were reported by Ottolina.²⁴³ Similar to sulfide oxidations the ee values of the products were highly influenced by the structural factors and further studies by Colonna and co-workers showed that secondary amines are converted to hydroxylamines **169** and nitrones **171** and **172**.²⁴⁴ PAMO and its M446G mutant (methionine to glycine) have been reported to oxidize amines. Interestingly the mutant was also able to convert indole into indigo,²⁴⁵ a phenomenon observed also recently for a new BVMO clone (B2 BVMO) derived from a metagenomic library.²⁴⁶

4.7. Miscellaneous Reactions

4.7.1. Enzymatic Baeyer–Villiger Oxidation of Aldehydes. The BV oxidation of an aldehyde can yield two different products, the corresponding acid or the ester of the corresponding alcohol and formic acid, also called formate (Figure 46). Depending on the steric and electronic nature of the aldehyde and the oxidizing reagent applied, different ratios of the two products are obtained. For example, electron-rich benzaldehyde derivatives give exclusively phenols when reacted with hydrogen peroxide under alkaline conditions in the so-called Dakin reaction.²⁴⁷ Several selenium-based catalysts showed high selectivities for acid production from benzaldehyde derivatives, and peracid-mediated oxidations of phenylacetaldehyde gave ~2:1 to 7:1 ratios of formate to acid depending on the reaction conditions applied.^{7c} An inverted preference for the oxidation of phenylacetaldehyde (2:1, acid/formate) with purified CHMO from *Acinetobacter* NCIMB 9871 was observed by Branchaud and Walsh.²²¹ Furthermore enzymatic oxidations of aliphatic aldehydes gave predominantly the acid, reflecting the preferential migration of the hydrogen over the aliphatic substituent.²²¹ HAPMO was shown to exclusively catalyze the oxidation of 4-hydroxybenzaldehyde (**172b**) to the corresponding formate derivative, with no acid being detectable in the reaction mixture.⁵⁰ In a subsequent study the group of van Berkel investigated the selectivity of HAPMO-mediated oxidation of fluorobenzaldehydes (**172c–j**). Most mono- and difluorobenzaldehydes were selectively oxidized to the corresponding formates, and for only a few substrates minor amounts of acid were detected too (Figure 47). These results complement nicely the chemical oxidations of electron-poor benzaldehydes, which give predominantly acids.²⁴⁸ In addition, the M446G PAMO mutant was shown to accept benzaldehyde as substrate; however the selectivity for this reaction was not reported.²⁴⁵

4.7.2. Epoxidations. Two reports on the epoxidation of olefins by BVMOs have been published in the literature so far.

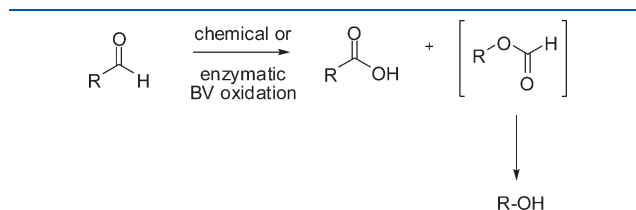


Figure 46. BV oxidation of aldehydes.

Substrate	R ₁	R ₂	R ₃	n	Biocatalyst	Yield (%)	Ratio 173 : 174	Reference
172a	H	H	H	1	CHMO _{Acineto} - purified	n. r.	65 : 35	221
172b	H	H	OH	0	HAPMO - purified	n. r.	0 : 100	50
172c	F	H	H	0	HAPMO - purified	88	5 : 95	248
172d	H	F	H	0	HAPMO - purified	83	18 : 82	248
172e	H	H	F	0	HAPMO - purified	75	0 : 100	248
172f	F	H	OH	0	HAPMO - purified	86	0 : 100	248
172g	H	F	OH	0	HAPMO - purified	91	0 : 100	248
172h	F	F	H	0	HAPMO - purified	25	27 : 100	248
172i	F	H	F	0	HAPMO - purified	64	5 : 95	248
172j	H	F	F	0	HAPMO - purified	31	0 : 100	248

^a n. r. = not reported.

Figure 47. BVMO-catalyzed oxidations of phenylacetaldehyde and substituted benzaldehydes.

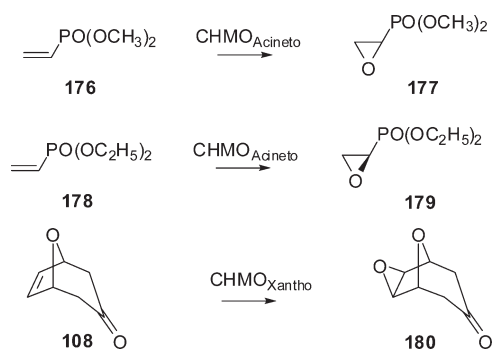


Figure 48. BVMO-mediated oxidations of olefins.

Screening of numerous unsaturated compounds with either electron-donating or electron-withdrawing groups adjacent to the olefin functionality led to the identification of dimethyl and diethyl vinyl phosphonates (**176** and **178**, Figure 48) as suitable substrates for BVMO-catalyzed epoxidations.²⁴⁹ Purified enzyme preparations of CHMO from *Acinetobacter* NCIMB 9871 yielded optically pure (*R*)-oxirans. Kinetic data for diethyl phosphonate illustrated the low affinity of CHMO_{Acineto} for the tested substrate ($K_m = 3$ mM) and explained the rather sluggish conversion of the electron-deficient olefin.²⁴⁹ The second example for a BVMO-mediated epoxidation reaction was observed by Mihovilovic and co-workers during a detailed substrate profiling with recombinant *E. coli* cells expressing a BVMO from *Xanthobacter* sp. ZLS.¹⁹⁹ The authors described the isolation of an epoxide derived from meso-bridged bicyclic ketone **108** in moderate yield as highlighted in Figure 48. Detailed control experiments with either noninduced cell suspensions or *E. coli* carrying a plasmid without the BVMO encoding gene verified that the epoxidation reaction could be attributed to the sole action of the BVMO. Interestingly the structurally closely related carbo-bridged derivative **109** gave the corresponding enantiopure (–)-lactone (Figure 29).¹⁹⁹

5. CRYSTAL STRUCTURES

5.1. Phenylacetone Monooxygenase

The first crystal structure of a BVMO, PAMO, was published by Mattevi and co-workers in 2004 (Figure 49).⁶⁵ The structure of the prototypical *Acinetobacter* CHMO was never forthcoming apparently due to the unstable nature of the enzyme. PAMO is a stable monomeric 62-kDa enzyme derived from the thermophilic bacterium *Thermobifida fusca* that catalyzes the conversion of phenylacetone to phenylacetate. It displays 40.3% homology to the *Acinetobacter* CHMO. The crystal structure of PAMO revealed two domains, a FAD-binding domain and a NADP-binding domain. In the absence of bound NADP, the NADP-binding site was predicted based on comparison with structures of flavin/NAD(P)H complexes with similar folding topology.

Crystallization of the protein with bound FAD allowed for a detailed analysis of the FAD-binding mode. The flavin ring was shown to be completely embedded by the protein and involved in a number of van der Waals and H-bond interactions with the protein. On the *si* side, the flavin made extensive van der Waals contacts with a cluster of conserved aromatic amino acids (W55, Y60, and Y72). Located on the *re* side of the flavin ring was R337, a strictly conserved residue among BVMOs. Previous mutagenesis studies²⁵⁰ had suggested that R337 stabilizes the negatively charged flavin-peroxide intermediate formed during BVMO catalysis. In the crystal structure of PAMO, R337 was located in front of the flavin C4a atom and adopted two side chain conformations that could possibly interact with the flavin-peroxide intermediate. Based on the observed flexibility of the R337 side chain, the authors predicted that R337 adopts two alternate conformations during BVMO catalysis: (i) an IN position (as observed in the crystal structure) involved in stabilization of the flavin-peroxide intermediate and (ii) an OUT position that allows positioning of NADPH adjacent to the flavin ring for cofactor reduction.

The crystal structure also revealed that a sequence motif characteristic of all BVMOs (FXGXXXHXXXW)₁₃₈ corresponds to a

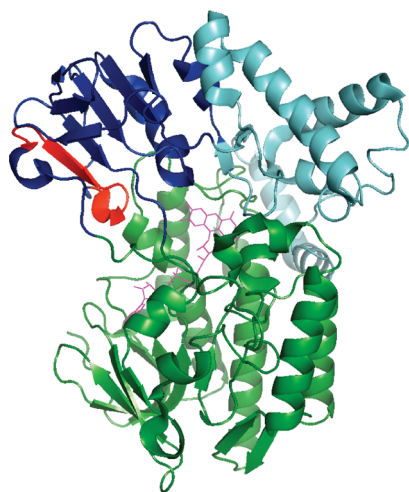


Figure 49. Ribbon diagram of PAMO crystal structure (PDB 1W4X). The FAD binding domain (residues 10–158 and 390–542) is shown in green, the NADP binding domain (residues 159–389) is shown in blue, the α -helical subdomain (220–340) within the NADP binding domain is shown in cyan, and fingerprint residues (167–177) are shown in red. The FAD cofactor is shown in purple.⁶⁵

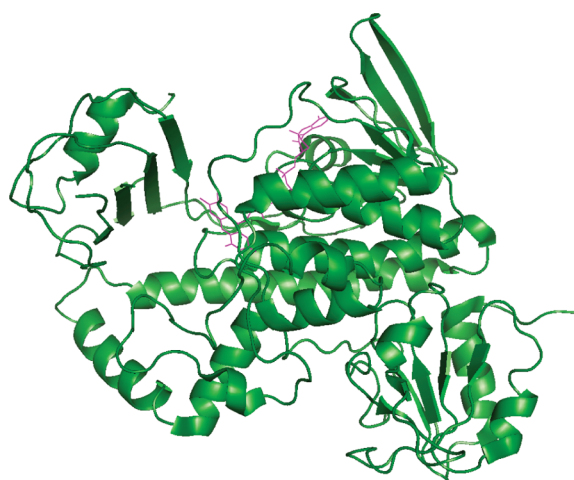


Figure 50. Overall structure of MtmOIV monomer.⁷¹ The FAD cofactor is shown in purple.

linker segment in PAMO that connects the FAD-binding domain to the NADP-binding domain. Mutagenesis of the histidine residue (H173 in PAMO) in other BVMOs had shown that this residue was crucial for catalysis and FAD binding.^{2,251} In the PAMO crystal structure, H173 is 16 Å from the flavin and approximately 10 Å from the NADP-binding site. Because of the location of the motif, it was deemed unlikely to have a direct role in catalysis. The authors speculated that the sequence motif residues take part in domain rotations and conformational changes that occur during the catalytic cycle.

5.2. Mithramycin Monooxygenase

MtmOIV is involved in mithramycin biosynthesis and performs an oxidative cleavage of the fourth ring of premithramycin B via a BV reaction. Unusual as this enzyme may be, it represents the first BVMO kinetically characterized with its natural substrate.²⁰ MtmOIV has very low sequence identity with other

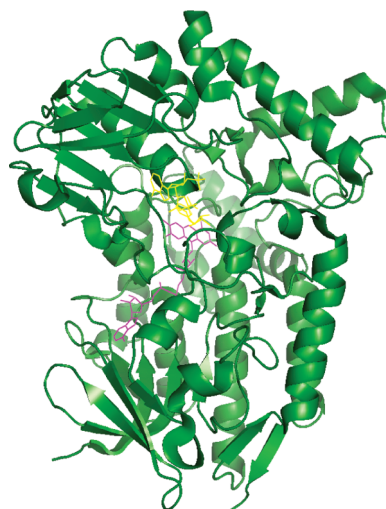


Figure 51. Overall structure of CHMO. The FAD cofactor is shown in purple and NADP is shown in yellow.⁶³

BVMOs (only 8% sequence identity with PAMO) and lacks the typical sequence motif of type 1 BVMOs. In 2009, the crystal structure of MtmOIV was solved with bound FAD (Figure 50).⁷¹ The overall structure of MtmOIV was found to be very different from the PAMO structure with a root-mean-square deviation of 5.78 Å for 282 aligned residues. MtmOIV is a dimer whereas PAMO is a monomeric enzyme. The active site of MtmOIV contains an Arg-52 residue above the flavin ring to possibly stabilize the peroxyflavin and Criegee intermediates. In PAMO, R337 was located on the *re* side of the flavin ring, while R52 was located on the *si* side of the flavin ring.

Whereas the PAMO structure resembles that of a disulfide oxidoreductase, MtmOIV was found to be highly similar both in sequence and in structure to PgaE, a FAD-dependent hydroxylase of the glucocorticoid receptor (GR₂) subfamily. Both MtmOIV and PgaE enzymes are classified as class A flavoprotein MOs.¹⁹ However, PgaE is a typical class A flavoprotein that requires a hydroperoxyflavin intermediate for its electrophilic aromatic substitution reaction, whereas, MtmOIV represents an atypical class A flavoprotein MO because it requires a peroxyflavin intermediate for its nucleophilic attack of the keto functionality in premithramycin B.

5.3. Cyclohexanone Monooxygenase

Recently, the first crystal structures of a CHMO and the first structures of a BVMO in the presence of both FAD and NADP⁺ were published (Figure 51).⁶³ This CHMO was derived from a soil actinomycete *Rhodococcus* sp. HI-31. The sequence identities to PAMO and the classical *Acinetobacter* CHMO are 43% and 57%, respectively. Structures of CHMO from *Rhodococcus* sp. strain HI-31 in complex with FAD and NADP⁺ were determined in two different crystal forms to a resolution of 2.3 and 2.2 Å. Two cocrystallization conditions provided two structures: CHMO_{open} and CHMO_{closed}. Similar to the PAMO structure, each CHMO structure contained a FAD binding domain, a NADP binding domain, and a helical domain comprising residues that make up the substrate binding pocket (Figure 51). CHMO_{open} and CHMO_{closed} were found to differ significantly in the relative orientation of the NADP domain (Figure 52) and in the conformation of a loop comprising residues 487–504. These

differences resulted in alternate nicotinamide cofactor binding modes and significant changes in the size and accessibility of the substrate binding pocket.

The CHMO_{closed} structure contained a well-defined active-site pocket with an enclosed cavity consisting of primarily hydrophobic residues (Figure 53A). In CHMO_{open}, the same pocket was enlarged and solvent accessible due to the movement of the mobile loop formed by residues 487–504 (Figure 53B). The significant rotation of the NADP domain effected large shifts in several active site residues including the strictly conserved R329 residue, which is equivalent to residue R337 in the PAMO enzyme. The conformation of R329 in the CHMO_{open} structure was found to be similar to the R337 “out” conformation observed in PAMO, in which the side chain shifts during catalysis to accommodate the cofactor binding. In contrast, flexing of loop 327–330 in the CHMO_{closed} structure caused R329 to adopt a novel “push” position. The side chain appeared to push the nicotinamide head deeper into the enzyme to potentially allow for stabilization of the peroxyflavin and Criegee intermediates. The CHMO_{closed} was thought to represent the conformation of the enzyme in the post-flavin-reduction state, and the CHMO_{open} structure represented the final step in the catalytic cycle, namely, NADP⁺ release.

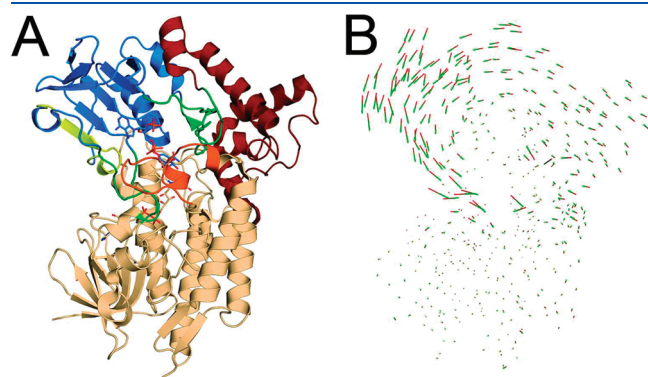


Figure 52. (A) Overall structure of the closed conformation of CHMO, colored by domain. The FAD-binding domain is shown in wheat, the NADPH-binding domain in blue, the helical domain in brown, the linker regions in green, the key mobile catalytic loop in orange, and the BVMO signature sequence in yellow. (B) Equivalent atom representation of the open and closed conformations of CHMO. After alignment of the structures using the FAD-binding domain, vectors were drawn between equivalent C-α atoms.⁶³

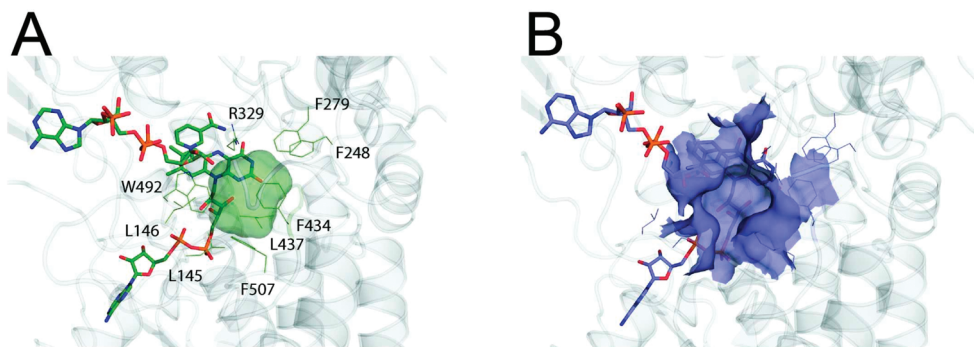


Figure 53. (A) The putative substrate binding cavity as seen in the closed conformation of CHMO, formed primarily by hydrophobic residues. (B) In comparison, the larger binding cavity in the open conformation caused by the movement of the loop formed by residues 487–504.⁶³

The NADP⁺-bound crystal structure of CHMO also revealed a novel role for the BVMO signature motif (FXGXXHXXXW) in cofactor binding. The BVMO signature sequence was shown to be anchored at each end by F160 and W170, which interact with hydrophobic pockets in the NADP binding domain (Figure 54). In the middle of the motif, the critical histidine residue (H166) formed a hydrogen bond to the backbone of one of the flexible linker segments (residues 381–386) responsible for connecting the FAD and NADP domains and positioning the NADP⁺ cofactor through steric interactions. Thus, the sequence motif was found to be important for BVMO catalysis due to its role in coordinating domain movements that allow for binding and positioning of the NADP⁺ cofactor.

5.4. 3,6-Diketocamphane Monooxygenase

The 3,6-DKCMO from *P. putida* NCIMB 10007 is a BVMO enzyme involved in the oxidative-degradation pathway of (–)-camphor. It was reported to be a loose trimeric complex containing an NADH dehydrogenase subunit and a homodimeric FMN-binding oxygenating component.⁷⁰ A recent publication describes how a noncrystallographic symmetry (NCS) exhaustive search was used to solve the crystal structure of this enzyme.²⁵² Though not discussed by the authors at the time of publication, this structure should provide some insight into which residues are involved in FMN and substrate binding.

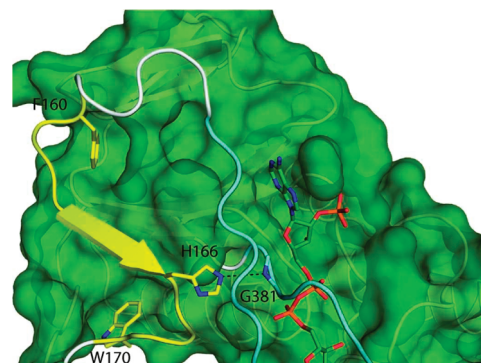


Figure 54. The BVMO signature sequence shown in yellow is anchored by F160 and W170, which interacts with hydrophobic pockets in the NADPH-binding domain. H166 is shown to be interacting with G381, part of a key linker (cyan) that connects the FAD- and NADPH-binding domains.⁶³

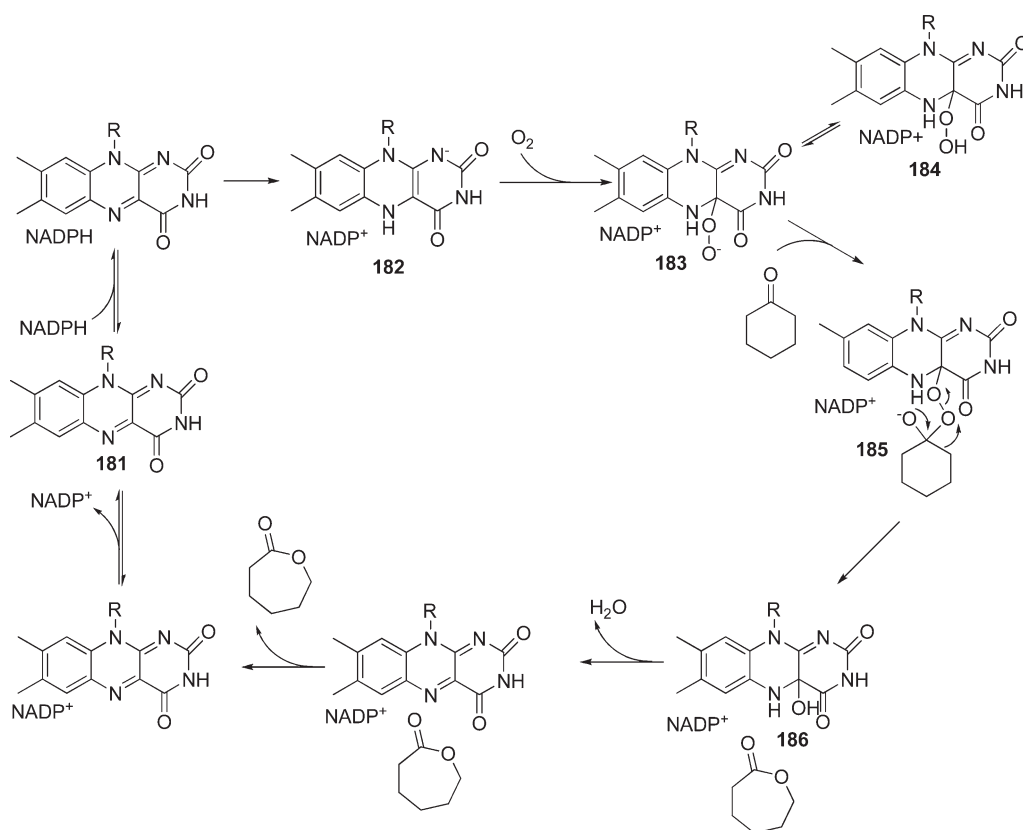


Figure 55. Proposed mechanism for the enzymatic oxidation of cyclohexanone with CHMO from *Acinetobacter*.²⁵³

6. MECHANISTIC STUDIES

6.1. Cyclohexanone Monooxygenase

The mechanism of CHMO-catalyzed oxidation of cyclohexanone has been shown by kinetic and spectroscopic data to proceed in a manner very similar to the chemical BV reaction. In the chemical reaction, addition of an oxidant, such as *m*-chloroperoxybenzoic acid, to the carbonyl group of a ketone creates a tetrahedral intermediate that undergoes “Criegee” rearrangement to yield the corresponding ester or lactone. With BVMOs, the tightly bound FAD molecule **181** is reduced by NADPH (Figure 55). The reduced flavin **182** then reacts with molecular oxygen to form a C4a-peroxyflavin intermediate **183**. This peroxyflavin intermediate plays the same role as the peracid in the conventional BV oxidation reaction, acting as the nucleophile in the nucleophilic attack of the carbonyl carbon of the ketone substrate. This produces the tetrahedral Criegee intermediate **185** that subsequently rearranges to give the C4a-hydroxyflavin **186** and ϵ -caprolactone. A molecule of water is spontaneously eliminated from the hydroxyflavin to generate the oxidized FAD. The NADPH, which is the first species to bind to the enzyme and react with the flavin, is held in the active site in the NADP⁺ form until the last step of the reaction cycle (Figure 55).

Ryerson, Ballou, and Walsh provided the first experimental evidence of this mechanism by performing steady-state and pre-steady-state kinetic analysis.²²⁰ Their studies with CHMO from *Acinetobacter* NCIMB 9871 showed that in the presence of NADP, a flavin–oxygen adduct was formed from the reaction of the reduced enzyme with oxygen. From its spectral properties, this flavin–oxygen adduct was speculated to be either an

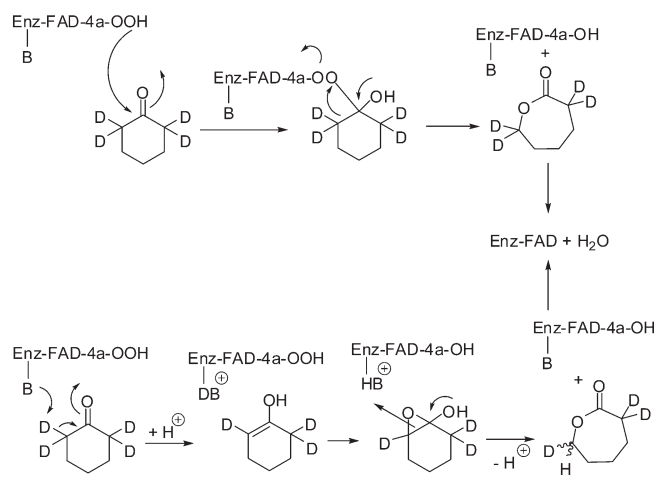


Figure 56. During the incubation of CHMO with [2,2,6,6-²H₄]cyclohexanone, no proton exchange was observed between the deuterated substrate and the protic solvent, which suggested that the ketone did not react through an enolate intermediate.¹⁰¹

enzyme-associated flavin C4a-peroxide **183** or an enzyme-associated flavin C4a-hydroperoxide **184**. At the time of publication, no kinetic or spectral evidence could distinguish between these two possibilities. However, the reaction of CHMO with [2,2,6,6-²H₄]cyclohexanone provided strong evidence for the peroxide mechanism. No proton exchange was observed between the deuterated substrate and the protic solvent, which suggested that the ketone did not react through an enolate intermediate (Figure 56).

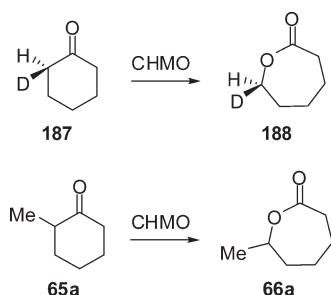


Figure 57. Oxidation of (2*R*)-[2-²H]₁cyclohexanone (top) proceeded with retention of configuration. Oxidation of 2-methylcyclohexanone (bottom) resulted in preferential migration of the more substituted α -carbon.^{152b}

Around the same time, Schwab and co-workers had also observed via NMR studies that [2,2,6,6-²H₄]cyclohexanone showed negligible loss of deuterium in the CHMO-catalyzed reaction.^{152a} They extended their NMR studies to investigate the stereochemistry of the enzyme-catalyzed BV reaction of cyclohexanone.^{152b} CHMO was incubated with (2*R*)-[2-²H]₁-cyclohexanone (187) and the product ϵ -caprolactone was analyzed for chirality at C-6 (Figure 57a). The enzyme-catalyzed reaction proceeded with retention of configuration, a well-known characteristic of the chemical BV reaction.⁷ Next, they investigated the regioselectivity of the CHMO-catalyzed oxidation of 2-methylcyclohexanone (Figure 57b). Similar to the chemical BVMO reaction,⁷ preferential migration of the more substituted center in 2-methylcyclohexanone (65a) was observed. The results of the stereochemical and regiochemical studies were consistent with a BV mechanism where the 4a-peroxy flavin 183 acts as the oxygen transfer agent.

More than a decade later, Sheng and co-workers were able to confirm that the flavin C4a-peroxide 183 was the active oxygenating agent toward cyclohexanone.²⁵³ They identified several reaction intermediates by spectral rapid kinetic studies and examined the dependence of their spectra on pH. When the reaction was carried out at pH 7.2, the first intermediate observed was the flavin C4a-peroxide 183 with maximum absorbance at 366 nm. In the presence of cyclohexanone, oxidized FAD and ϵ -caprolactone product were formed rapidly. In the absence of cyclohexanone, the C4a-peroxide 183 was in slow protonic equilibrium with the flavin C4a-hydroperoxide 184 with an observed pK_a of 8.4. The protonated species 184 was unreactive with cyclohexanone and could be interconverted with the deprotonated form by altering the pH.

6.1.1. Active-Site Models for Predicting Stereoselectivity. In the absence of a CHMO crystal structure before 2004, several groups developed active-site models based on experimental results to explain the enzyme's stereoselectivity. Alphand and Furstoss developed a simple model of the active site based on the highly regio- and enantioselective oxidation of bicyclic butanones.²⁰⁵ For the two CHMOs from *Acinetobacter* NCIMB 9871 and *Acinetobacter* TD63, the oxidation of bicyclo[3.2.0]hept-2-en-6-one (122a) afforded a 1:1 ratio of two regioisomeric lactones (123a and 124a) with ee values as high as 95%. Oxidation of the (*S*)-enantiomer afforded lactone 123a via "normal" BV type oxygen insertion between the more substituted carbon atom and the carbonyl group. Oxidation of the (*R*)-enantiomer afforded the "abnormal" lactone 124a. Their model depicted the active site as a cube with the peroxidic bond

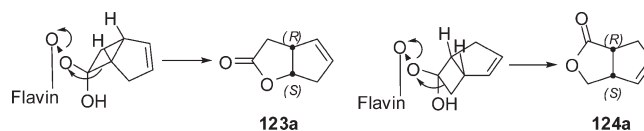


Figure 58. Taschner's configurational model for the oxidation of bicyclo[3.2.0]hept-2-en-6-one.

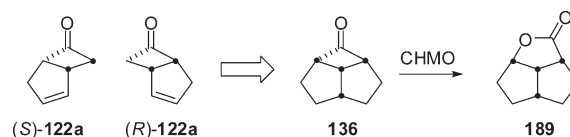


Figure 59. Tricyclic ketone 136 mimicked the superimposition of enantiomeric bicyclic ketones. Migratory bonds shown with dashed lines.

immobile at the bottom. The rest of the molecule was allowed to rotate around the O–C(1) bond to position the migrating C–C bond of the peroxidic intermediate antiperiplanar to the peroxidic bond and to a nonbonded electron pair of the hydroxide group.²⁵⁴ For the (*S*)-enantiomer, they proposed that steric interactions between the enzyme and cyclohexyl ring of the ketone prevented the hydroxyperoxide intermediate from adopting more than one configuration. For the (*R*)-enantiomer, two configurations were possible, and it was proposed that electronic interactions with the active site might favor the formation of the configuration leading to the "abnormal" lactone. A refined active-site model was later published based on results obtained for the BVMO oxidations of 2-substituted cyclohexanones.¹⁵⁹

Some 4 years earlier, Taschner developed a predictive stereochemical model based on the CHMO-catalyzed oxidation of 4-substituted cyclohexanones.²⁵⁵ This model proposed two potential tetrahedral intermediates for the attachment of the hydroperoxyflavin to the cyclohexanone: axial attachment of the hydroperoxide with the resultant equatorial hydroxyl oriented up relative to the flavin and equatorial attachment of the hydroperoxide with the resultant axial hydroxyl pointing down toward the flavin (Figure 58). Migration of the bond α to the carbonyl that is antiperiplanar to the leaving group in either of these two intermediates produces the lactone with the observed *S*-configuration. The equatorial attachment of the hydroperoxide with the hydroxyl down, where it might possibly hydrogen bond with the C4 carbonyl of the isoalloxazine, was favored. This predictive model also explained the absolute stereochemistry and migratory preference for the oxidation of bicyclo[3.2.0]hept-2-en-6-one (122a) (Figure 58).

Kelly and co-workers also examined the regiodivergent oxidation of bicyclo[3.2.0]hept-2-en-6-one (122a) and proposed that the enantiomeric ketones were bound at the active site of CHMO with their cyclobutanone rings in overlapping positions (Figure 59).^{202a} Consequently the peroxide, hydroxy group, and migrating bond for both intermediates adopted essentially identical positions. To test their hypothesis, they constructed tricyclic ketone 136, which mimicked the superimposition of the enantiomeric ketones. BV oxidation of the ketone with CHMO from *Acinetobacter* afforded the tricyclic lactone 189 as a single enantiomer with absolute stereochemistry as predicted. Given the known facial selectivity for nucleophilic attack and migrating bond of the tricyclic ketone, it was possible to assign the

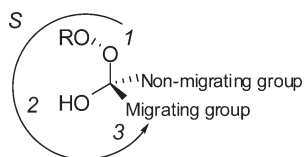


Figure 60. Absolute configuration of the CHMO Criegee intermediate.

stereochemistry of the Criegee intermediate. A model for assigning stereochemistry was developed that gave priority of the migrating group over the nonmigrating group. With the priority rules $OO > O > \text{migrating} > \text{nonmigrating}$, the CHMO Criegee intermediate was assigned an (*S*)-configuration (Figure 60).

Following these early models, Ottolina proposed an active-site model for CHMO from *Acinetobacter* NCIMB 9871 based on cubic descriptors to predict the stereochemistry for BV oxidations of ketones and sulfoxidation of sulfides.^{232b} In addition, Stewart and Kayser proposed a diamond lattice model of allowed alkyl substituent positions for 2-, 3-, and 4-substituted cyclohexanone oxidations.^{160b}

6.2. Phenylacetone Monooxygenase

Following the elucidation of the PAMO crystal structure, the kinetic properties of wild-type PAMO were reported.²⁵⁶ Despite having 40% sequence identity with CHMO_{Acinetobacter}, significant differences were observed between the two enzymes' kinetic mechanisms. For example, the formation of the C4a-peroxyflavin intermediate was significantly slower for PAMO ($k_{\text{ox}} = 870 \text{ mM}^{-1} \text{ s}^{-1}$) than CHMO ($k_{\text{ox}} \geq 5000 \text{ mM}^{-1} \text{ s}^{-1}$). The oxygenated form of PAMO had an absorption maximum of 380 nm, which suggested direct formation of the C4a-hydroperoxyflavin intermediate. In contrast, the C4a-peroxyflavin intermediate was formed first in CHMO, then slowly protonated to afford the C4a-hydroperoxyflavin intermediate. The rate-limiting step of PAMO catalysis occurred in the penultimate step of the mechanism and involved the decay of an unidentified enzyme intermediate. The rate-limiting step of the CHMO catalysis was determined to be a conformational change occurring prior to the release of NADP^+ .

Two mutants, R337A and R337K, were also investigated to determine the role of residue R337 in BVMO catalysis. Previous mutagenesis studies²⁵⁰ had suggested R337 stabilizes the negatively charged C4a-peroxyflavin formed during BVMO catalysis. In the present kinetic study, mutation of the arginine residue to both alanine and lysine resulted in a drastic decrease in flavin reduction rate compared with wild-type enzyme, while the binding of NADPH was not affected. The oxygenation rates of the NADPH-reduced mutants were ~ 20 -fold less than the NADPH-reduced wild-type enzyme, providing evidence that the arginine residue was important but not essential for C4a-peroxyflavin formation. No product was obtained for the mutants when phenylacetone or benzylmethyl sulfide were used as substrates, which suggested that the arginine may play a role in forming a productive substrate–C4a-peroxyflavin complex.

Zambianchi and co-workers proposed that the R337 residue may also play a role in the pH dependence of PAMO enantioselectivity.²⁵⁷ For the kinetic resolution of racemic 2-phenylpropionaldehyde to (*S*)-formic acid 1-phenylethyl ester, the enantiomeric ratio (*E* value) decreased from 26.5 to 16.7 when the pH was raised from 7 to 10. The pH profile of the

E values resembled a simple acid/base titration curve and a pK_a of 9.2 was determined. Theoretical pK_a values were calculated for all of the amino acids in PAMO, and five residues were identified with values close to pK_a 9.2. Of these five residues, R337 was in closest proximity to the flavin (7.53 Å). The authors proposed that the protonation state of R337 may have influenced the stereochemical arrangement of the transition state for 2-phenylpropionaldehyde oxidation. A pH effect was also observed for the PAMO-catalyzed sulfoxidation of thioanisole. However, in this case, ee values increased when pH increased from 6 to 10 and a pK_a of 7.8 was determined. All of the PAMO amino acids with theoretical pK_a values close to this value were located too far from the flavin to influence selectivity. Instead, the authors proposed that the observed pH effect was possibly related to the pK_a of the FAD–C4a-hydroperoxide/FAD–C4a-peroxide equilibrium, whereby the FAD–C4a-hydroperoxide intermediate influenced the geometry of the electrophilic attack to the sulfur atom.

7. PROTEIN ENGINEERING

When the three-dimensional structure of an enzyme is unavailable, many researchers turn to directed evolution²⁵⁸ to improve the enzyme. Directed evolution involves random mutagenesis of the entire protein and screening of mutant libraries for the improved property. Consequently, the success of any directed evolution study relies heavily on the efficiency of the screening method in place. In addition to the GC-based screening method utilized by Reetz and co-workers (discussed below),²⁵⁹ several groups have developed methods for screening BVMO catalysis in high-throughput. Littlechild and co-workers developed the first colorimetric assay based on pH change associated with esterase-catalyzed cleavage of the BMVO-produced lactone.²⁶⁰ Furstoss and co-workers developed the first fluorescence assay based on umbelliferone release.²⁶¹ Reymond and co-workers also developed a fluorescence assay based on umbelliferone release from cyclic and noncyclic 2-coumaroyloxy ketones.²⁶²

7.1. Directed Evolution

Reetz and co-workers pioneered the first directed evolution study of a BVMO that improved the enantioselectivity of CHMO from *Acinetobacter* sp. NCIMB 9871 for the desymmetrization of 4-hydroxycyclohexanone (**83i**).²⁵⁹ The wild-type enzyme afforded the (*R*)-lactone **84i** in 9% ee, which spontaneously rearranged to (*R*)-**85i** with retention of stereochemistry. In the absence of a three-dimensional structure of CHMO at that time, error-prone PCR was used as the mutagenesis method to create a library of random mutants. A total of 10 000 mutants were screened in 96-well format, using a GC-based screening system to determine the ee values of the products. In the first round of directed evolution, four mutants were obtained with improved ee in favor of the (*R*)-enantiomer (Figure 61). The best result was obtained with the double mutant L426P/A541V. In addition, four mutants were obtained with improved (*S*)-selectivity. In this case, the best result (79% ee) was obtained with the single mutant F432S. To further improve the (*R*)-selectivity, hits from the first round were used as individual templates for second rounds of random mutagenesis by error-prone PCR. A triple mutant (E292G/L435Q/T464A) was identified from the L143F library with an ee value of 90% in favor of the (*R*)-enantiomer (Figure 61).

The mutants were then compared with wild-type CHMO for the desymmetrization of 4-methoxycyclohexanone (**83b**).

R-C1CCCCC1=O (83) \longrightarrow R-C1CCC2C(C1)OCC2=O (84) \longrightarrow R-C1CCC2C(C1)OCC2=O (85i)
 if R = OH

Substrate	R	Biocatalyst	Mutation	% ee	Configuration	Reference
83a	Me	CPMO	n. a.	46	R	271
		A1-A10	F450I	92	R	271
		B1-A10	F156L, G157F	91	R	271
83b	OMe	CHMO _{Acineto}	n. a.	78	S	259
		2-D19-E6	L143F, E292G, L435Q, T464A	25	R	259
		1-K2-F5	F432S	98.6	S	259
83i	OH	CHMO _{Acineto}	n. a.	9	R	259
		1-C2-B7	F432Y, K500R	34	R	259
		1-F1-F5	L143F	40	R	259
		1-E12-B5	F432I	49	R	259
		1-H7-F4	L426P, A541V	54	R	259
		2-D19-E6	L143F, E292G, L435Q, T464A	90	R	259
		1-D10	F432Y	17	R	259
		4-D11	F432P	72	R	259
		1-H3-C9	L220Q, P428S, T433A	18	S	259
		1-F4-B9	D41N, F505Y	46	S	259
		1-K6-G2	K78E, F432S	78	S	259
		1-K2-F5	F432S	79	S	259
		1-C8	F432G	17	S	259
83r	OAc	CPMO	n. a.	5	S	271
		A1-A3	G449S, F450Y	59	R	271
		B1-G4	F156N, G157Y	90	R	271

Figure 61. Mutant BVMO-mediated oxidations of 4-substituted cyclohexanones.

Wild-type CHMO afforded the (*S*)-enantiomer with an ee value of 78% whereas the best (*R*)-selective mutant (L143F/E292G/L435Q/T464A) afforded the (*R*)-enantiomer with 25% ee. The best (*S*)-selective mutant, F432S mutant, afforded the (*S*)-enantiomer with an ee value of 98.6%. Amino acid F432 was determined to be a hot spot for enantioselectivity and was subjected to saturation mutagenesis to introduce all 20 amino acids. A screen of this library with 4-hydroxycyclohexanone afforded two (*R*)-selective mutants (F432Y and F432P) with ee values of 17% and 72%, respectively. Two (*S*)-selective mutants were identified as F432G (17% ee) and F432S (79% ee), the latter having an identical amino acid sequence to the mutant identified in the first round of directed evolution (Figure 61). In a later publication, a diamond model was used to explain the possible effect of the 432 serine residue on the enantioselectivity of 4-hydroxycyclohexanone.^{33b}

As an extension of these studies, the original library of 10 000 mutants was screened for enantioselective oxidation of thioethers.²⁶³ For the oxidation of methyl-*p*-methylbenzyl thioether (**158r**), wild-type CHMO afforded the (*R*)-sulfoxide with an ee of 14%. The screen identified more than 20 hits having >85% ee and five mutants with ee > 95% were sequenced (Table 2). Two (*R*)-selective mutants were identified, each containing one amino acid substitution. The D384H mutant afforded the (*R*)-sulfoxide with an ee of 98.9%. Mutant F432S, previously identified as an (*S*)-selective mutant for 4-substituted cyclohexanones, also afforded the (*R*)-sulfoxide with high ee

(98.7%). Three (*S*)-selective mutants were also identified, the best of which contained four amino acid changes and afforded the (*S*)-sulfoxide with an ee of 99.7% (Figure 62). In all cases, achiral sulfone was formed as a side product (5–27%) due to over-oxidation of the sulfoxide. Error-prone PCR was performed on the gene for the best (*S*)-selective mutant, and a library of 1600 clones was screened for high (*S*)-selectivity and minimal sulfone formation. A hit from this screen contained three of the four mutations of the parent gene in addition to three new amino acid substitutions (Q92R/Y132C/P169L/F246N/V361A/T415A). The (*S*)-sulfoxide was formed in 99.8% ee, and sulfone formation was less than 5% compared with 26.6% obtained with the parent enzyme. The best mutants were then compared with wild-type CHMO for the oxidation of ethylphenyl thioether (**158f**). Wild-type CHMO afforded the (*R*)-enantiomer with an ee value of 47%. The double mutant F16L/F277S afforded the (*R*)-enantiomer with 88% ee, and the K229I/L248P mutant afforded the (*S*)-enantiomer with 98.9% ee (Figure 62).

In a later study, mutants from the directed evolution of CHMO were further assessed for their substrate specificity and stereoselectivity toward a library of structurally diverse ketones.²⁶⁴ Overall, the most interesting results were obtained with mutants containing amino acid substitutions at the 432 position as well as the double mutant (L426P/A541V). For example, two exo tricyclic ketones that were not substrates for the wild-type enzyme were recognized by two double mutants

Substrate	R ₁	R ₂	Biocatalyst	Mutation	% ee	Configuration	Reference
158b	Ph	Me	PAMO	n. a.	41	R	245
				M446G	93	R	245
158f	Ph	Et	CHMO _{Acineto}	n. a.	47	R	263
			1-J8-C5	F16L, F277S	88	R	263
			1-C5-H3	K229I, L248P	98.9	S	263
			PAMO	n. a.	6	S	245
				M446G	95	R	245
158g	<i>p</i> -Me-Ph	Me	PAMO	n. a.	6	R	245
				M446G	92	R	245
158r	<i>p</i> -Me-Bn	Me	CHMO _{Acineto}	n. a.	14	R	263
			1-D10-F6	D384H	98.9	R	263
			1-K15-C1	F432S	98.7	R	263
			1-C5-H3	K229I, L248P	98.1	S	263
			1-H8-A1	Y132C, F246I, V361A, T415A	99.7	S	263
			1-J8-C5	F16L, F277S	95.2	S	263
			2-K11-F11	Q92R, Y132C, P169L, F246N, V361A, T415A	99.8	S	263

^a n. a. = not applicable.

Figure 62. Mutant BVMO-mediated oxidations of sulfides.

(L426P/A541V and F432Y/K500R) as well as the single mutant F432S.

7.2. Phenylacetone Monooxygenase

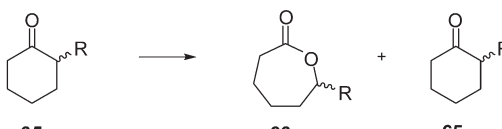
The elucidation of the PAMO crystal structure in 2004 enabled researchers to use rationally designed and semirationally designed approaches to alter the substrate specificity of BVMOs. The PAMO wild-type enzyme was known for its thermal stability but also for its low substrate acceptance and poor enantioselectivity. A homology model of wild-type CHMO was constructed based on the crystal structure of PAMO.²⁶⁵ An overlap of the CHMO homology model and PAMO structure revealed the presence of a bulge in the loop near the active site of PAMO consisting of three residues: S441, A442, and L443. To minimize this bulge and thus expand substrate acceptance, three variants were designed based on the deletion of A442 (P1), A442 and L443 (P2), and S441 and A442 (P3). Interestingly, residue F432, the “hot-spot” position in CHMO, was located at the same position as the L443 residue in PAMO.

The double deletion variant P3 afforded the most significant changes in substrate acceptance and enantioselectivity. Less than 10% conversion was observed for the oxidation of 2-phenylcyclohexanone (**65k**) with wild-type PAMO. The *E* value of the reaction was estimated to be 1.2 in favor of the (*S*)-enantiomer (Figure 63). Variant P3 oxidized the ketone with an *E* value of 100 in favor of the (*R*)-enantiomer. In addition, the mutant P3 enzyme converted 2-benzylcyclohexanone (**65l**) with an *E* value >200 in favor of the (*R*)-product. This substrate was not recognized by wild-type enzyme or the P1 and P2 variants. The results obtained with mutant P3 were very similar to those obtained with wild-type CHMO. Modeling of the 2-phenylcyclohexanone oxidation showed that the wild-type PAMO did not provide enough space in the active site to position 2-phenylcyclohexanone in the form of a Criegee intermediate due to the presence of the bulge. In contrast, a model of the PAMO variant P3 contained the Criegee

intermediate arising from 2-phenylcyclohexanone in a reactive geometry with two H-bonds originating from the catalytic R337.

The substrate specificity of wild-type PAMO and its mutants was found to be limited to ketones bearing a phenyl group near the carbonyl function. In an effort to expand the substrate acceptance to aliphatic ketones, a rational approach was used based on the homology model of CPMO from *Comamonas* sp. strain NCIMB 9872.²⁴⁵ In the active site, the two enzymes were found to differ by three residues: Q152, L153, and M446 in PAMO were aligned with F156, G157, and G453 in CPMO, respectively. A single (M446G), double (Q152F/L153G) and triple mutant (Q152F/L153G/M446G) were prepared and evaluated for their activity toward cyclopentanone, cyclohexanone, and phenylacetone. The double and triple mutants were inactive toward all three substrates. The single mutant was also inactive toward cyclopentanone and cyclohexanone but showed activity toward phenylacetone. Compared with wild-type PAMO, the affinity of M446G toward phenylacetone decreased 10-fold. However, several substrates that were not oxidized by the wild-type enzyme were converted by the M446G mutant. For example, low activity was observed with 3,5-dimethylcyclohexanone, indole, benzaldehyde, *N*-methylbenzylamine and isopropylphenyl thioether. In addition, the M446G mutation increased the enantioselectivity of the enzyme toward aromatic sulfides (Figure 62).

Another approach to broadening the substrate acceptance of PAMO involved saturation mutagenesis at all four positions of the 441–444 loop.²⁶⁶ Alignment of the amino acid sequence of PAMO with seven other BVMOs identified a limited number of amino acids at these four positions. The libraries were designed with reduced amino acid alphabets to randomly incorporate these amino acids at each position. Screening toward 2-phenylcyclohexanone (**65k**) identified a variant with four amino acid substitutions (A, W, Y, T) with an *E* value of 70 in favor of the (*R*)-enantiomer. Variants affording the (*S*)-enantiomer were only slightly more enantioselective than the wild-type enzyme. The best (*R*)-selective mutants also oxidized 2-(4-chlorophenyl)cyclohexanone (**65p**) with high enantioselectivity



Substrate	R	Biocatalyst	Mutation	<i>E</i> -value	Configuration	Reference
65k	Ph	PAMO	n. a.	1.2	S	265
		P1	A442 deletion	35	R	265
		P2	A442 and L443 deletion	23	R	265
		P3	S441 and A442 deletion	100	R	265
			S441A, A442W, L443Y, S444T	70	R	266
			P440F	12		267
65l	Bn	PAMO	n. a.	n. d.		265
		P3	S441 and A442 deletion	>200	R	265
			P440F	48		267
65p	<i>p</i> -Cl-Ph	PAMO	n. a.	n. d.		266
			S441A, A442W, L443Y, S444T	>200	R	266
			P440F	7		267
65q	<i>p</i> -Me-Ph	PAMO	P440F	117		267
			Q93N, P94D	1.5		268
65a	Me	PAMO	P440F	95		267
			Q93N, P94D	25		268
65c	Et	PAMO	P440F	26		267
			Q93N, P94D	50		268
65d	<i>n</i> -Pr	PAMO	P440F	145		267
			Q93N, P94D	68		268
65g	<i>n</i> -Bu	PAMO	P440F	39		267
			Q93N, P94D	40		268
65f	allyl	PAMO	P440F	102		267
			Q93N, P94D	42		268
65e	<i>iso</i> -Pr	PAMO	P440F	>200		267
			Q93N, P94D	>200		268
65r	<i>cyclo</i> -Hex	PAMO	P440F	145		267
			Q93N, P94D	25		268
65o	CH ₂ CH ₂ CN	PAMO	P440F	91		267
			Q93N, P94D	55		268

^a n. a. = not applicable; ^b n. d. = not determined.

Figure 63. Mutant BVMO-mediated kinetic resolution of 2-substituted cyclohexanones.

(*E* > 200) but could not convert 2-(4-methylphenyl)cyclohexanone (65q).

In addition to the (441, 442, 443, 444) site, four new randomization sites were chosen for mutagenesis based on docking studies of phenylacetone with wild-type PAMO.²⁶⁷ Unfortunately, a screen toward 2-phenylcyclohexanone (65k) failed to identify any hits from these libraries. The focus then switched to two conserved proline residues, P437 and P440. These “second sphere” residues were selected for saturation mutagenesis to determine whether the introduction of a more “flexible” amino acid would alter the spatial position of neighboring active site residues and broaden the substrate specificity of the enzyme. A screen for oxidation of 2-ethylcyclohexanone (65c) produced no hits from the P437 library. Ten hits were obtained for the P440 library and the most active mutants were further characterized for their enantioselectivity toward a library of 2-substituted cyclohexanones (Figure 63). The most active mutant

from the initial screen, P440F, converted all additional ketones tested with *E* values ranging from 7 for 2-(4-chlorophenyl)cyclohexanone (65p) to >200 for 2-isopropylcyclohexanone (65e) (Figure 63). For the kinetic resolution of bicyclo[3.2.0]hept-2-en-6-one, all P440 mutants afforded ~50:50 ratios of “normal” and “abnormal” lactones with ee values between 87% and 99%. This was similar to previous results obtained for the oxidation by wild-type CHMO.²⁰⁵ In comparison, the wild-type PAMO favored formation of the “normal” lactone as the (1*S*,*S**R*)-enantiomer with 86% ee and also produced some “abnormal” lactone as the (1*S*,*S**R*)-enantiomer with 63% ee.

The most recent approach to broadening the substrate scope of PAMO involved the of distal mutations in the enzyme to alter the structure of the binding pocket via allosterically induced domain movements.²⁶⁸ A strategy was devised to introduce attractive interactions between an α -helix (A91–E95) and the Y56–Y60 loop (FAD-binding domain), which would result in the movement of the loop

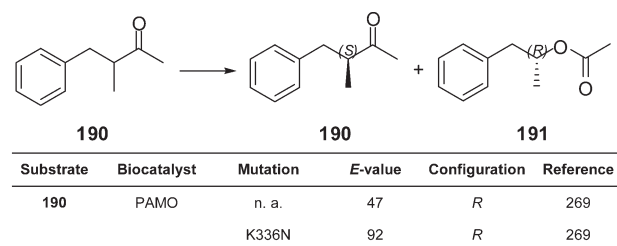


Figure 64. Mutant PAMO-catalyzed kinetic resolutions of racemic 3-methyl-4-phenylbutan-2-one.

segment W177–E180 (NADP-binding domain) due to strong attractive contacts between the two domains.

Mutant Q93/P94 was chosen as the randomization site, and saturation mutagenesis was performed using NDT codon degeneracy to encode 12 amino acids simultaneously at each position. The library was screened for the oxidation of 2-ethylcyclohexanone (**65c**) and two double mutants were identified with activity: Q93V/P94F and Q93N/P94D. The more active variant, Q93N/P94D, was tested further for the kinetic resolution of 2-substituted cyclohexanones and for the desymmetrization of 4-substituted cyclohexanones. With the exception of 2-(4-methylphenyl)-cyclohexanone ($E = 1.5$), the variant resolved all 2-substituted cyclohexanones (Figure 63) with good ($E = 25$) to excellent enantioselectivity ($E > 200$) and was highly enantioselective for the desymmetrization of 4-substituted cyclohexanones.

Surprisingly the two single mutants, Q93N and P94D, showed only traces of activity with 2-methylcyclohexanone (**65a**) and were inactive toward the other substrates. In addition, no active variants were found when the single saturation mutagenesis libraries were generated at positions 93 and 94. The above results suggested a strong cooperative effect between the two amino acid substitutions. Molecular dynamics simulations of the wild-type PAMO and Q93N/P94D revealed a novel salt bridge between residues D94 and R59, anchoring the rigid helix structure to the loops Y56–Y60 and W177–E180. This salt bridge was not found in the inactive single mutant P94D, likely due to steric interactions that would result between residues R59 and Q93.

Superposition of the average structures of wild-type PAMO and Q93N/P94D mutant revealed a backbone rotation of the NADP-binding domain for the double mutant. This resulted in displacements between 3 and 5 Å of both distal and active site residues. The active site in the wild-type PAMO appeared as an enclosed cavity, while the active site of the double mutant appeared more solvent accessible and more capable of binding large substrates. The conformational differences between wild-type PAMO and the double mutant Q93N/P94D were comparable to the observed structural differences between the open and closed crystalline forms of CHMO.⁶³

Very recently, rational design studies to investigate the coenzyme specificity of PAMO also identified mutations that improved the enantioselectivity of the enzyme.²⁶⁹ The amino acid K336 in PAMO was targeted based on previous results showing that the equivalent residue K439 in HAPMO was important for coenzyme specificity.²⁵⁰ The K336N mutant did not have any beneficial effects on coenzyme specificity but increased the enantioselectivity from $E = 47$ to 92 for the kinetic resolution of racemic 3-methyl-4-phenylbutan-2-one (Figure 64).

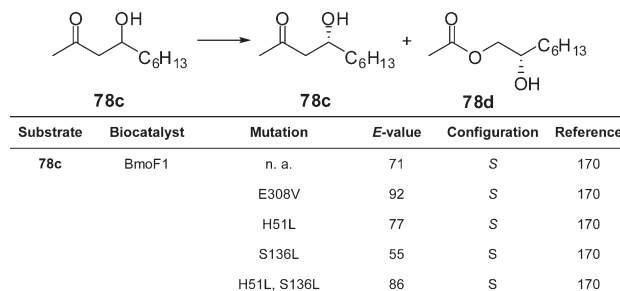


Figure 65. Mutant BmoF1-catalyzed kinetic resolutions of 4-hydroxy-2-decanone.

7.3. Cyclopentanone Monooxygenase

To increase the enantioselectivity of CPMO toward 4-substituted cyclohexanones, a semirational approach was used based on the “hot-spots” identified as important for enantioselectivity for CHMO.⁴⁰ An overlay of the CHMO and CPMO homology models showed that residues 143 and 432 in CHMO corresponded to positions 156 and 450 in CPMO. The neighboring residues of these amino acids, 157 and 449, were also located and selected to generate two focused libraries using a restricted CASTing approach (CAST = complete active site saturation test).²⁷⁰ In CASTing, two or three amino acids in the binding pocket are simultaneously randomized to create a library of mutants. Primers for saturation mutagenesis are usually designed with NNK degenerate codons (N represents any of the four nucleotides (A, T, G, or C); K represents T or G) to encode all 20 amino acids at the selected position. In an effort to reduce screening, the restricted approach used NDT degeneracy (N = A, T, G, or C; D = A, G or T; T = T), to encode only 12 amino acids at each position.

The two libraries were screened toward 4-methylcyclohexanone (**83a**), 4-acetoxycyclohexanone (**83r**), and 4-*tert*-butylcyclohexanone (**83f**). No conversion of the latter ketone was observed for wild-type or mutant enzymes. Oxidation of 4-methylcyclohexanone (**83a**) by the wild-type CPMO enzyme afforded the (*R*)-enantiomer in 46% ee. From library A, a single amino acid substitution resulted in the largest enhancement in enantioselectivity. Variant F450I afforded the (*R*)-enantiomer in 92% ee (Figure 61).²⁷¹ A similar improvement was observed with the double mutant F156L/G157F from library B, which afforded the (*R*)-enantiomer in 91% ee. For the oxidation of 4-acetoxycyclohexanone (**83r**), the majority of the mutants afforded the (*R*)-enantiomer, whereas wild-type CPMO afforded the (*S*)-enantiomer with 5% ee. The most significant change in enantioselectivity was observed with mutant F156N/G157Y, which afforded the (*R*)-enantiomer in 90% ee. The presence of two hydrophilic residues suggested a possible interaction with the polar OAc group. However, this variant was also found to be (*R*)-selective toward 4-substituted cyclohexanones with nonpolar groups (ethyl, propyl) whereas the wild-type was (*S*)-selective.²⁷¹

7.4. BmoF1 from *Pseudomonas fluorescens* DSM 50106

The enantioselectivity of the BVMO from *P. fluorescens* DSM 50106 was improved by directed evolution for the kinetic resolution of 4-hydroxy-2-decanone (**78c**).¹⁷⁰ Error-prone PCR was used to create a mutant library of ~3500 clones. The mutants were first screened for activity toward racemic 4-hydroxy-2-decanone using a microtiter-plate screening system. The

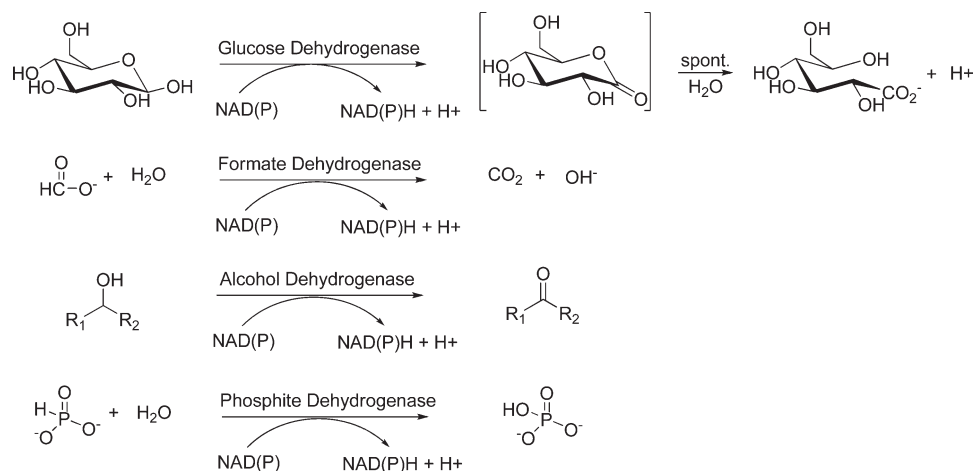


Figure 66. Enzymatic NAD(P)H cofactor recycling systems.

active clones were then screened for activity toward (*R*)- and (*S*)-4-hydroxy-2-decanone in parallel assays to identify clones with higher apparent enantioselectivity. A total of 170 clones from the enantioselectivity screen were used in small scale-up reactions with racemic ketone to determine the true *E* value of the reaction by chiral GC. Approximately 20 clones were identified with improved enantioselectivity. The wild-type enzyme catalyzed the kinetic resolution of 4-hydroxy-2-decanone (**78c**) with an *E* of 71, and an *E* of 92 was obtained for the best mutant E308V (Figure 65). All improved mutants from the first round of directed evolution were randomly recombined and screening identified a double mutant (H51L/S136L) showing higher enantioselectivity (*E* = 88) than the original single mutations H51L (*E* = 77) and S136L (*E* = 55). A homology model based on the crystal structure of PAMO showed that all improved mutants were located far from the active site. In general, directed evolution experiments targeting the entire protein often discover distant mutations for statistical reasons: there are more amino acids distant to the active site than close; thus a greater number of distant mutations are generated.²⁷²

8. COFACTOR RECYCLING STRATEGIES

With the emergence of isolated BVMO enzymes, a number of strategies have been developed for cofactor regeneration in BVMO-catalyzed reactions. For flavin reduction, the hydride source is either of the two forms of nicotinamide adenine dinucleotide cofactor: NADPH or NADH. Cofactor regeneration is necessary to avoid high costs associated with stoichiometric use of these cofactors. For enzymatic regeneration of cofactors, a second enzyme is added to recycle the oxidized NAD(P)⁺ back to NAD(P)H. NADPH regeneration systems commonly used in BVMO-catalyzed oxidations are glucose-6-phosphate/glucose-6-phosphate dehydrogenase,^{113,176,273} formate/NADP-dependent formate dehydrogenase,²⁷⁴ and 2-propanol/ADH from *Thermoanaerobium brockii* (Figure 66).^{209f,275} More recently, a phosphite dehydrogenase (PTDH) was studied for NADPH regeneration as both the free protein and a fusion protein that had been covalently linked to a BVMO protein (see section 8.3 for more details). The advantages and limitations of all NADPH regeneration systems have been extensively discussed in previous reviews.^{276,277}

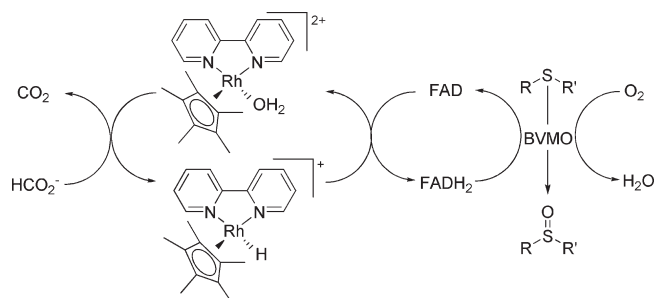


Figure 67. Organorhodium complex $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ for FAD regeneration in the BVMO-catalyzed oxidation of sulfides.²⁷⁸

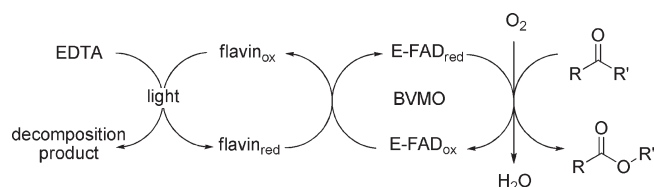


Figure 68. Light reduction of FAD in the presence of ethylenediaminetetraacetate.²⁷⁹

8.1. Chemical Strategies

Ottolina and co-workers utilized the organorhodium complex $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ for FAD regeneration in the BVMO-catalyzed oxidation of sulfides and sulfoxides.²⁷⁸ Treatment of $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$ with sodium formate resulted in the formation of $[\text{Cp}^*\text{Rh}(\text{bpy})\text{H}]^+$ which acted as an electron donor to reduce the FAD cofactor (Figure 67). However, with all four BVMOs investigated (PAMO, CHMO, HAPMO, and EtaA), product ee's and reaction conversions were significantly lower than values obtained for oxidations carried out in the presence of NADP and the glucose-6-phosphate/glucose-6-phosphate dehydrogenase recycling system.

8.2. Photochemical Strategies

Hollman and co-workers demonstrated the direct photochemical regeneration of FAD in the PAMO-catalyzed oxidation of selected ketones (Figure 68).²⁷⁹ In this strategy, light reduced

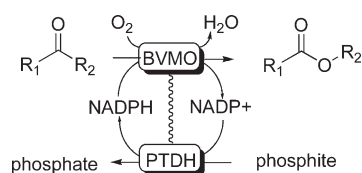


Figure 69. Coenzyme regeneration by fusion enzyme.²⁸¹

FAD in the presence of the electron donor ethylenediaminetetraacetate (EDTA), and a catalytic amount of NADPH was required for substrate turnover. Optical purities of the photo-enzymatic reaction products matched those obtained with conventional cofactor regeneration. However, the catalytic activity of the enzyme was significantly reduced in the light-driven reaction compared with a reaction using NADPH regeneration by a coupled enzyme. The major limitations of the light-driven regeneration system were identified as unproductive oxidative uncoupling of the light-driven flavin reduction reaction from the enzymatic oxygenation reaction and slow electron-transfer between free and PAMO-bound FAD due to poor active-site accessibility.²⁸⁰

8.3. Fusion Engineering

In this approach, the BVMO was covalently linked to phosphite dehydrogenase to create a bifunctional fusion enzyme capable of BVMO oxidation and cofactor regeneration (Figure 69).²⁸¹ Phosphite dehydrogenase (PTDH) converts the inexpensive phosphite to phosphate with simultaneous reduction of NADP. Fusing together the BVMO and PTDH simplified enzyme production and isolation. In this study, three BVMOs (CHMO, CPMO, and PAMO) were linked to PTDH. For each of the fused BVMOs (CRE-CHMO, CRE-CPMO, CRE-PAMO), the catalytic activity of the BVMO subunits was similar to the nonfused enzyme. A decrease in affinity for phosphite was observed for the fused PDH compared with the nonfused enzyme. The stereoselectivity of fused CHMO and CPMO was examined for the desymmetrization of prochiral substrates and for the regiodivergent oxidation of fused bicyclic ketones. In most cases, the stereochemistry was comparable to the unfused proteins. For a handful of substrates, the enantioselectivity was slightly altered for the fused proteins. Surprisingly, both CRE-CHMO and CRE-CPMO converted a substrate not accepted by the unfused protein. In a subsequent publication,²⁸² a second generation of fused proteins was generated with BVMOs linked to a thermostable PTDH mutant developed by Zhao and co-workers.²⁸³ In comparison to wild-type, the mutant PTDH showed >7000-fold longer lifetime at 45 °C. Not surprisingly, the PTDH subunit of the second generation fused proteins showed higher thermostability than the PTDH subunit of the first generation proteins. After a reaction time of six hours, the oxidation of 4-methylcyclohexanone with first generation CRE-CHMO stopped at 40% conversion. No inactivation of the second generation CRE2-CHMO was observed within the same time period, and 80% conversion of the ketone was observed after 24 h.

9. BIOPROCESS DEVELOPMENT

9.1. Modeling

In an effort to guide process design for BVMO catalysis, modeling has been utilized to optimize reaction conditions for both whole-cell and isolated CHMO-catalyzed ketone oxidations.

Hogan and Woodley modeled the oxidation of 4-methylcyclohexanone with isolated CHMO using ADH from *T. brockii* for enzymatic regeneration of NADPH.²⁸⁴ Their model optimized the concentrations of both enzymes and cofactor to achieve acceptable reaction rates and product yield.

Woodley and co-workers modeled the kinetics of the oxidation of bicyclo[3.2.0]hept-2-en-6-one with whole cell CHMO to investigate potential benefits of adopting an *in situ* product removal technique.²⁸⁵ Due to product inhibition, the final product concentration of the initial process was limited to 3.5 g L⁻¹. Their process model predicted a maximum achievable product concentration of 11.5 g L⁻¹ by incorporating the *in situ* product removal technique.

Next Woodley and co-workers examined three main process limitations on the productivity of a whole cell CHMO catalyzed ketone oxidation: oxygen supply, product removal, and biocatalyst longevity.²⁸⁶ Modeling showed that the process productivity was very dependent upon biocatalyst concentration and consequently limited by oxygen supply rate when fermentation and biocatalytic conversion were carried out in the same bioreactor.

In addition to modeling, Woodley and co-workers demonstrated recently an innovative application of flow cytometry to monitor the effects of substrate and product concentrations on whole cells expressing *Acinetobacter* CHMO.²⁸⁷ High substrate concentrations (above 3 g L⁻¹) resulted in rapid cell damage (within 30 min, 73% cell viability) and were considerably more harmful than high product concentrations (cell viability 30 min postbiocatalysis initiation remained at 85% at up to 10 g L⁻¹ product concentration). In contrast, the product concentration affected substantially the cell viability over time and the ability of the whole cells to carry out the BVMO reaction.

9.2. Studies toward Industrial Scale Baeyer–Villiger Monooxygenase Oxidations

The use of BVMOs in large-scale applications has been largely limited by enzyme stability and substrate and product inhibition. Consequently, several strategies have been developed to improve enzyme stability and to maintain substrate and product concentrations below inhibitory levels.

9.2.1. Immobilization. Walsh and co-workers first reported the use of immobilized CHMO for the oxidation of cyclic ketones.¹¹³ Isolated CHMO from *Acinetobacter* NCIMB 9871 and glucose-6-phosphate dehydrogenase (G-6-PDH, for cofactor regeneration) were both immobilized in polyacrylamide gel (PAN 1000) and used in 1 L scale oxidations lasting between 5 and 10 days. For the oxidation of 2-norbornanone the residual activities of the recovered immobilized enzymes were 77% for CHMO and 80% for G-6-PDH after 5 days.

CHMO isolated from *E. coli* TOP10 [pQR239] and alcohol dehydrogenase from *T. brockii* (ADHTB) were immobilized on Eupergit C and used for the oxidation of thioanisole and bicyclo[3.2.0]hept-2-en-6-one.²⁷⁵ For the oxidation of thioanisole, the immobilized enzymes could be used for up to 16 cycles with complete substrate conversion (5 g L⁻¹) at each cycle. For bicyclo[3.2.0]hept-2-en-6-one, complete substrate conversion (5 g L⁻¹) was obtained up to three cycles.

In another study regarding immobilization of whole cells, encapsulation of *E. coli* with CPMO in polyelectrolyte complex (PEC) capsules improved storage stability of the cells at 4 °C.²⁸⁸ The CPMO-PEC capsules maintained their initial activity after 96 h, whereas the activity of the free cells decreased to 56%. Substrate conversions were similar for the oxidation of

8-oxabicyclo[3.2.1]oct-6-en-3-one using free and encapsulated cells. However, no data was provided regarding biocatalyst reuse.

9.2.2. Substrate Feeding. Inhibitory substrate and product concentrations of 0.2–0.4 g L⁻¹ and 4.5–5 g L⁻¹, respectively, were determined for the oxidation of bicyclo[3.2.0]hept-2-en-6-one with whole cell biocatalyst *E. coli* TOP10 [pQR239] expressing CHMO from *Acinetobacter* NCIMB 9871.²⁸⁹ A continuous substrate feeding strategy, aimed at maintaining the substrate concentration below inhibitory level, was used in a 55 L oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones.²⁹⁰ Due to product inhibition, a maximum of 3.5 g L⁻¹ (~200 g) of the combined lactones was produced, and the overall process yield of product on reactant was 85%. The process was later scaled to 200 L and resulted in 4.5 g L⁻¹ product formation.²⁹¹

A continuous substrate feeding approach was also applied to the oxidation of bicyclo[3.2.0]hept-2-en-6-one with CHMO isolated from *E. coli* TOP10 [pQR239] and immobilized on Eupergit C.²⁷⁵ In absence of substrate feeding, low enantiomeric purity of the “normal” lactone product was observed ($\leq 83\%$). With continuous substrate feeding, up to 10 g L⁻¹ of the substrate could be oxidized, and both regioisomeric lactone products were obtained with high ee ($\geq 95\%$).

9.2.3. Resins for Substrate Feeding or Product Removal. One strategy for controlling substrate/product inhibition of biocatalysts is resin-based *in situ* substrate feeding and product removal (SFPR).²⁹² Both the substrate and product can be released or adsorbed onto a resin, thus, avoiding the substrate and product accumulation in the aqueous phase. This approach was applied to the BMVO oxidation of bicyclo[3.2.0]hept-2-en-6-one with resting whole cells of a recombinant *E. coli* strain (TOP10 (pQR239) overexpressing CHMO from *Acinetobacter* NCIMB 9871.²⁹³ With a combination of increased cell concentration, improved aeration, and the addition of the adsorbent resin Optipore L-493, the biotransformation of bicyclo[3.2.0]hept-2-en-6-one could be improved from levels of 1 g L⁻¹ (9.3 mM) to 20 g L⁻¹ (185 mM). In the presence of 10 g of Optipore L-493 resin, 2 g of bicycloheptanone was successfully converted to a mixture of the two expected lactones (ee > 98%) at an overall yield of 83%.

In a subsequent publication,²⁹⁴ the Optipore L-493 resin was used in a special bubble column reactor to improve oxygenation, pH control, and glycerol feeding. Twenty-five grams (0.23 M) of *rac*-bicyclo[3.2.0]hept-2-en-6-one could be totally transformed using a 1-L vessel with a volumetric productivity of ~1 g L⁻¹ h⁻¹ (7.7 mmol L⁻¹ h⁻¹). As shown before, the two corresponding regioisomeric lactones were obtained in excellent enantiomeric purity (ee > 98%) and high preparative yield (84%). Under similar process conditions, biotransformation of 25 g of (–)-(1*S*,5*R*)-bicyclo[3.2.0]hept-2-en-6-one afforded enantiopure (–)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one in >99% ee and 84% yield.²⁹⁵ Recently, the same group published a very detailed protocol for the production of enantiopure (–)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one using the SFPR method.²⁹⁶ Further process optimization allowed for the very first near kilogram scale demonstration of a BVMO oxidation.²⁹⁷ Nine hundred grams of *rac*-bicyclo[3.2.0]hept-2-en-6-one was processed in a 50 L reactor at a substrate concentration of about 25 g L⁻¹. This reactor provided a volumetric productivity as high as 1.02 g of lactone L⁻¹ h⁻¹ (136 U L⁻¹) and a nonoptimized isolated product yield of nearly 60% was obtained.

Mihovilovic and co-workers applied the SFPR concept to the oxidation of three ketones with recombinant cells producing

CPMO from *Comamonas* NCIMB 9872.²⁹⁸ With nonbound substrate, the oxidation of 4-methylcyclohexanone with growing cells was limited to a concentration of 30 mM (3.36 g L⁻¹). When 4-methylcyclohexanone was adsorbed to Lewatit VPOC 1163 resin, the oxidation of 10 g L⁻¹ of ketone with growing CPMO cells proceeded to 77% conversion. With a combination of “nongrowing” cells and resin, the oxidation of 15 g L⁻¹ of 4-methylcyclohexanone went to 86% conversion. Similarly, the oxidation of 15 g L⁻¹ of *rac*-3-methylcyclohexanone went to 90% conversion. In addition, the concentration of 8-oxabicyclo[3.2.1]oct-6-en-3-one could be increased from 2.0 to 5 g L⁻¹ using “nongrowing” cells and the SFPR concept.

Recently, the *in situ* SFPR strategy was applied to the kinetic resolution of 3-phenyl-2-butanone with HAPMO from *P. putida* JD1.²⁹⁹ HAPMO showed excellent enantioselectivity toward 3-phenyl-2-butanone with *E* > 100 but was inhibited by concentrations >10 mM (>1.5 g L⁻¹) of both substrate and product. On 50 mL scale, the concentration of 3-phenyl-2-butanone could be increased from 1.4 to >26 mM using substrate-to-resin ratios of 1:2 for Optipore L-493 resin or 1:5 for Lewatit resin. On larger scale (500 mL), higher conversions were obtained with the Lewatit resin.

Overall, the *in situ* SFPR strategy appears to be an effective strategy for controlling substrate/product inhibition for recombinant whole cell BVMO biotransformations. For the oxidation of 1,3-dithiane with isolated CHMO enzyme, Zambianchi and co-workers found that the resin-adsorbed substrate could not be used since the enzyme also adsorbed on the resin.³⁰⁰ With whole cells of CHMO, better reaction conversions were observed for shaken flask experiments with resin-adsorbed substrate. However, when the oxidation was carried out in a bioreactor, productivity was higher for reactions containing the nonbound substrate.

9.2.4. Biphasic Reactions. Another approach for avoiding substrate and product inhibition is performing the reaction in a biphasic system using a water nonmiscible organic solvent. The organic phase acts as a substrate reservoir and as an extraction medium for *in situ* removal of product from the aqueous phase. For the oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one, substrate concentrations greater than 1 g L⁻¹ inhibited whole cells of PAMO mutant P3.³⁰¹ With diethylphthalate as the organic phase, substrate levels could be increased to 3 g L⁻¹. With isolated enzyme, substrate levels could be increased to 5 g L⁻¹ when the oxidation was carried out in a 1:1 mixture of cyclohexane and buffer containing 0.1% (v/v) Tween-20. Similarly, the kinetic resolution of 2-phenylcyclohexanone with isolated enzyme proceeded at 5 g L⁻¹ using a 1:1 mixture of MTBE and buffer containing 0.1% (v/v) Tween-20.

Lau and co-workers described the first bioproduction of lauryl lactone (C₁₂) from cyclododecanone using the recombinant CPDMO and a biphasic fermentation medium.³⁰² In a two-phase semicontinuous reactor, cells were grown in a 1 L aqueous phase by continuously feeding nutrients and inducer in order to maintain the enzyme expression. The organic phase consisted of 375 mL of hexadecane containing 120 mM of cyclododecanone. Ten to sixteen grams of lauryl lactone was produced with the two-phase semicontinuous reactor system compared with 2.4 g of lactone produced via batch mode.

9.2.5. Growing versus Nongrowing Cells. Whole cell BV oxidations performed under growing conditions require low concentrations of substrate or product to prevent inhibition of cell growth. One strategy for increasing volume productivity of whole cell biotransformations involves the use of nongrowing cells in place of growing cells.³⁰³ Walton and Stewart examined

the oxidation of cyclohexanone with growing and nongrowing *E. coli* cells overexpressing *Acinetobacter* sp. CHMO.³⁰⁴ Under growing conditions, cells were grown in M9 medium for 11.25 h, then cyclohexanone was added to a final concentration of 10 mM. A volume productivity of 0.047 g of ϵ -caprolactone L⁻¹ h⁻¹ was obtained, and the total lactone yield was 2.7 g. For the transformation with nongrowing cells, cells were pregrown in rich medium then harvested and resuspended in M9 medium lacking a nitrogen source. To 3 L of resuspended cells, cyclohexanone was added to a final concentration of 30 mM. The volume productivity for cyclohexanone oxidation was 0.79 g of ϵ -caprolactone L⁻¹ h⁻¹ (20-fold improvement over growing cells) with a total lactone yield of 27 g. In the latter process, product isolation was facilitated by passing the reaction mixture through a column of XAD-4 resin and washing the resin with organic solvent to recover the ϵ -caprolactone.

10. CONCLUSIONS AND OUTLOOK

10.1. Applications

The enzymatic BV oxidation technology has been advanced considerably since its initial discovery more than 60 years ago. Since the 1990s, detailed substrate screening studies identified BVMOs as valuable biocatalysts for the enantioselective oxidations of aliphatic, aromatic, and alicyclic ketones, and therefore this technology has allowed access to chiral synthons that are otherwise difficult to prepare chemically. These intermediates have been applied to the total syntheses of natural products, and these examples highlighted the efficiency and superiority over the chemical BV oxidation. Recent developments in molecular biology and protein engineering have allowed for the production of large quantities of BVMOs, and “tailored” proteins, although limited, with regard to substrate acceptance and enantioselectivity have also been made possible. Moreover, successful strategies have been introduced to solve the problem of cofactor recycling. However, despite these achievements, the real breakthrough of the BV biooxidation in industry has still not been realized. With the current trend to replace traditional chemical operations with environmentally benign processes in the chemical industry, the time is ripe for the enzymatic BV oxidation. A major challenge is the identification of reactions for the synthesis of target lactones in which the BV biooxidation is superior to the conventional chemical oxidation with regard to efficiency and cost saving. On the other hand, biocatalysis is most useful when there is no equivalent chemical route. The case of “abnormal” lactones in the biotransformation using bicyclo[3.2.0]hept-2-en-6-one is an example. Clearly, more target substrates of this type need to be identified. For reasons of safety and efficacy, the requirement for single enantiopure compounds in drug synthesis is in increasing demand.³⁰⁵ Hence, it is a prime time to seek out new targets for BV biooxidations.

On the “designer” theme, Kayser and Stewart had advanced the idea of producing the *Acinetobacter* CHMO in a dry yeast form in order to make the bioreagent more user-friendly for chemists, although biocatalytic reactions can be conducted quite readily in a chemical laboratory without specialized equipment. An appropriate host, such as a “generally regarded as safe (GRAS)” organism that would withstand desiccation or simply provide the requisite enzyme in a stable immobilized form would further improve the ease of applications.

10.2. Crystal Structures and Rational Protein Engineering

The recently solved crystal structures of CHMO in complex with both FAD and NADP⁺ have provided valuable insight into

the conformational changes required for BVMO catalysis. Going forward, structures of BVMOs in complex with substrates or transition-state analogs of the Criegee intermediate would dramatically enhance our understanding of (1) the role of the invariant active-site arginine in the formation of a productive substrate–C4a-peroxyflavin complex, (2) formation and stabilization of the Criegee intermediate during catalysis, (3) the nature of the substrate-binding pocket when a given BVMO exhibits a broad substrate spectrum ranging from large to small ring size, and (4) the influence of other possible protein folds in catalysis or stability of those BVMOs that have an added N-terminal extension, for example, CPDMO and HAPMO (~60–147 amino acids), in comparison to the prototypical CHMO or PAMO sequences. Clearly, more snapshots of the conformational changes and domain movements underlying the dynamic catalysis events would be warranted. Unfortunately, to which side NADPH binds the flavin (*re* versus *si*) has not been reconciled from the available kinetic data and structure information.

Since type 1 BVMOs are dependent on the relatively expensive NADPH as cofactor, there was the desire to change the specificity toward NADH, for example, the case of HAPMO.^{250,306} However, a simple amino acid substitution was not enough since a highly active mutant was compromised by low affinity characterized by a high K_m value toward NADH. A formidable challenge is ahead if one were to mutate a greater number of residues that would maintain the proper hydrogen bonding and electrostatic interactions and maintain accessibility to the flavin as noted by a recent work on a FMO from *Methylophaga* sp. strain SK1.³⁰⁷

10.3. Directed Evolution to Relieve Substrate or Product Inhibition

To date, the protein engineering studies with BVMOs have focused on improving enantioselectivity or increasing substrate acceptance. If one is actually going to develop a viable process with a BVMO (typically process goals are 100 g/L substrate, 5 g/L biocatalyst), protein engineering is first needed to relieve substrate and product inhibition. If the mechanism of substrate and product inhibition is unknown, several rounds of error prone PCR may first be required, followed by generation and screening of focused libraries based on hits from the initial screens. At each round, screening would involve the incubation of the mutant libraries with increasing concentrations of substrate and product and then measuring residual activities. Likely, a combination of protein engineering and process engineering (SFPR, biphasic medium, etc.) would be necessary to meet the requirements of a manufacturing process.

10.4. Pathways of Opportunities

In addition to the available biodiversity of putative BVMOs, the pathways these putative genes reside in the respective genomes are also potentially valuable in both academic and industrial pursuits. A recent example is assignment of the *Acinetobacter* CHMO-related “CMO” (Bpro 5565) of *Polaromonas* sp. strain JS666³⁰⁸ to be responsible for the first step of degradation of *cis*-dichloroethene to DCE epoxide, thus expanding the repertoire of BVMO substrates. Although the *Trichosporon* genome sequence is not yet available, a potential BVMO was postulated to be responsible for the lactonization of a macrocyclic compound, zearalenone, a potent estrogenic mycotoxin produced by several *Fusarium* species that colonize agricultural crops such as maize.³⁰⁹ Zearalenone is subsequently hydrolyzed by another unknown lactonase/esterase to a nonestrogenic

metabolite. Which BVMO and corresponding ring-opening enzyme that will recognize these macrocyclic compounds are exciting prospects.

In the classical CHMO degradation pathway (Figure 3) and others that have been elucidated, the full gene complement, normally organized in a cluster, would allow the production of valuable dicarboxylic acids such as adipic acid or 1,12-diacid, the latter from cyclododecanol/cyclododecanone metabolism. These compounds are building blocks for nylon 6,6 and polyamide synthesis. However, considerable metabolic engineering efforts would be needed to make this type of biological processes viable. Nonetheless these pathway genes, in various combinations, are available for exploration.

Along with the pathway genes come the promoter sequences and regulatory elements that drive gene expression. ChnR, belonging to the AraC/XylS family of transcriptional activators, was the first regulator identified and characterized for the degradation of monocyclic cycloparaffin compounds in the *Acinetobacter* sp. strain NCIMB 9871.^{21b} Inducible expression of cloned *chnB* expressing CHMO in *E. coli* was dependent on the presence of *chnR*. However, to date, there has been a paucity of information on the ways that the cycloalkanol/cycloalkanone degradative pathways are regulated. Interestingly, although not surprisingly, the *Acinetobacter* sp. *chnB* promoter together with the transcriptional regulator *chnR* were used to construct gene cassettes to control expression levels of targeted proteins or other metabolic engineering purposes in Gram-negative bacteria.³¹⁰

All in all, the BVMO technology not only is about what it can do for green chemistry but has also contributed to the understanding of the many intricacies that embody these flavoproteins. The context of the BVMO-encoding gene in its genomic space is another open opportunity to embrace. Needless to say, the future is both bright and green.

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ABBREVIATIONS

ACMO	acetone monooxygenase
ADH	alcohol dehydrogenase
BV	Baeyer–Villiger
BVMO	Baeyer–Villiger monooxygenase
CDMO	cyclodecanone monooxygenase
CHMO	cyclohexanone monooxygenase
CPDMO	cyclopentadecanone monooxygenase
CPMO	cyclopentanone monooxygenase
CRE	coenzyme regenerating enzyme
DKCMO	diketocamphane monooxygenase
DPNH	reduced diphosphopyridine nucleotide
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetate
ee	enantiomeric excess
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FMO	flavin-containing monooxygenase
G-6-PDH	glucose-6-phosphate dehydrogenase
HAPMO	4-hydroxyacetophenone monooxygenase
MKMO	methylketone monooxygenase
MMKMO	monocyclic monoterpene ketone monooxygenase
MO	monooxygenase
MTBE	methyl <i>tert</i> -butyl ether
NAD ⁺	β -nicotinamide adenine dinucleotide
NADH	reduced β -nicotinamide adenine dinucleotide
NADP ⁺	β -nicotinamide adenine dinucleotide phosphate
NADPH	reduced β -nicotinamide adenine dinucleotide phosphate
OTEMO	2-oxo- Δ^3 -4,5,5-trimethylcyclopentenylacetic acid monooxygenase
<i>P. fluorescens</i>	<i>Pseudomonas fluorescens</i>
<i>P. putida</i>	<i>Pseudomonas putida</i>
<i>P. veronii</i>	<i>Pseudomonas veronii</i>

PAMO	phenylacetone monooxygenase
PCR	polymerase chain reaction
PEC	polyelectrolyte complex
PTDH	phosphite dehydrogenase
Ptl	pentalenolactone
SFPR	substrate feeding and product removal
SMO	steroid monooxygenase
TIM	triosephosphate isomerase

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NOTE ADDED IN PROOF

After submission of the original manuscript, several references appeared concerning the contents of this review. Pertinent to section 4.1.1, Liu and coauthors reported a novel metabolic product of dehydroepiandrosterone (DHEA), 15 α -hydroxy-17 α -oxa-D-homoandrost-4-ene-3,17-dione, for the first time using a new fungal strain *Penicillium griseopurpureum* Smith.³¹¹ The group of Swizer investigated the transformation of steroidal C-17 ketones with *Beauveria bassiana* KCH 1065 and found that only substrates bearing a 11 α -hydroxyl group were oxidized to 11 α -hydroxy ring-D δ -lactones.³¹² Relevant to section 4.5.3, Bornscheuer and coauthors investigated the kinetic resolution of N-protected β -amino ketones using recombinant whole cells of *E. coli* expressing a number of cloned BVMOs.³¹³ E values >200 were obtained for linear aliphatic and linear-branched aliphatic 4-amino-2-ketones/5-amino-3-ketones and one aryl-aliphatic 4-amino-2-ketone. The use of ionic liquids as cosolvents to improve the enantioselectivity of PAMO-catalyzed oxidation of racemic benzylketones was investigated by Gotor and coauthors.³¹⁴ The most significant improvement (20-fold) was observed for the oxidation of (\pm)-3-(*m*-trifluoromethylphenyl) butan-2-one. The E value increased from 4 (aqueous buffer) to 111 when the oxidation was performed in 10 vol % of ionic liquid Ammoeng 102. Furthermore, the use of ionic liquids facilitated an increase in substrate concentration compared with the kinetic resolution performed in aqueous media (relevant to section 9.2). Increasing the substrate concentration for BVMO reactions is an important development for industrial scale applications. Relevant to section 4.6, Gotor and co-workers reported on the oxidation of heteroaryl sulfides, cyclohexyl alkyl sulfides, and cyclic and linear aliphatic sulfides by three BVMOs: HAPMO, PAMO, and the PAMO mutant M446G.³¹⁵ Overall, HAPMO afforded the highest enantiomeric excess values (96% to >99% ee) and conversions for all substrate classes tested. On protein engineering (relevant to section 7), Opperman and Reetz reported the rational design of *Acinetobacter* sp. NCIMB 9871 CHMO mutants with improved oxidative stability and thermostability.³¹⁶ Methionine and cysteine residues of CHMO were targeted for site-directed mutagenesis due to the tendency of these sulfur-containing amino acids to readily undergo oxidation. All methionine and cysteine residues were mutated to small hydrophobic residues (isoleucine, leucine, and alanine) found in either PAMO or CHMO from *Rhodococcus* and then screened in parallel for hydrogen peroxide stability and thermostability. A C376L mutation afforded the best improvement in oxidative stability, while a M400I mutation resulted in the largest increase in thermal stability. Recombination of all improved mutants identified in the initial screening experiments afforded two CHMO mutants, M15 and M16, with significantly increased oxidation stability and

thermostability. While the wild-type CHMO was completely inactivated in the presence of 5 mM H₂O₂, both mutants retained >40% residual activity when incubated for 3 h in 100 mM H₂O₂. In addition, T_{50}^{10} values (the temperature at which half of the protein irreversibly denatured after a 10 min incubation) for M15 and M16 were increased by 3–7 °C (40.5 °C (wild-type CHMO) to 47.3 and 43.4 °C, respectively). Lack of a stable *Acinetobacter* CHMO was one reason why this prototypical BVMO was never crystallized. Relevant to section 9.2, Secundo and coauthors investigated the activity and stability of PAMO and CHMO in water-miscible organic solvents.³¹⁷ For PAMO, a 5-fold increase in specific activity was observed with 20% methanol. In addition, PAMO was found to be very stable in 20% methanol and retained 93% of its initial activity after incubation for 24 h. In contrast, CHMO exhibited a 1.2-fold increase in specific activity in 2% methanol and was fully inactivated after 24 h in 5% methanol. A greater number of ionic bridges in PAMO versus the predicted CHMO structure was postulated to contribute to the added stability of PAMO. After submission of this original manuscript, two additional review articles covering recent advances in BVMO technology^{318a,b} and a comprehensive review on the applications of BVMOs in organic synthesis^{318c} were also published. This is an attestation of growing interest and fast moving pace of the BVMO technology.