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‘Designer reagents’ recombinant microorganisms: new and powerful tools for organic synthesis

Margaret M. Kayser

Department of Physical Sciences, University of New Brunswick, Canada E2L 4L5

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E-mail address: kayser@unb.ca

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1. Introduction

Enzymes' ability to discriminate between enantiomers of racemic substrates and to desymmetrize *meso* compounds has been recognized for a long time and led to their application to asymmetric synthesis.[1] It is only recently, however, that enzyme-catalyzed biotransformations started to attract the attention of non-specialists. Technical problems associated with enzyme isolation, purification, and handling discouraged chemists from considering them as 'useful' reagents for organic synthesis. Partially purified enzymatic extracts, powders, and whole-cell catalyzed reactions were regarded with skepticism since it was not clear what enzyme or enzymes contributed to the formation of the product(s) in these transformations. In a chemist's eyes these transformations resembled more witchcraft than science. Ideally, a 'true' chemical reagent has a formula; and the reaction it performs has a mechanism, and is predictable, general and reproducible. The same concerns did not seem to hamper industrial chemists since enzyme- and whole-cell-catalyzed processes have been important in many industries for centuries. With the focus on preparation of single, valuable products fermentation protocols were developed and compounds such as L-sorbose, L-malic acid, D-aspartic acid, 2-keto-L-gulonic acid have been prepared in industry via whole-cell catalyzed biotransformations for many decades.[2]

Today's target molecules are almost always asymmetric and require 'asymmetric tools' for their construction; the pressure to find tools that are enantioselective, efficient, and environmentally acceptable compelled chemists to revisit biocatalysts as reagents for organic synthesis. Within a decade biocatalysis became an established field of research for chemists, principally because of rapid developments in molecular engineering, which provided them with an access to a range of interesting bioreagents. Much is due also to the success of lipases. Lipases were, and still are, the most frequently used biocatalysts in organic synthesis. These relatively stable, easy to handle enzymes do not require cofactors and many are commercially available. They hydrolyze esters in water but are also active in organic solvents where they carry out esterifications instead of hydrolyses, frequently in a highly enantioselective manner.[3] Acceptance and widespread use of lipases as reagents by a growing number of chemists paved the way for other biocatalysts into the repertoire of 'asymmetric tools' in organic laboratories.

Several excellent books,[4] reviews,[5] and dedicated special issues[6] trace the growth of application of biocatalysis and biocatalysts in organic synthesis over the last decade.[4] This report does not attempt to review the diversity of biotransformations that are presently used, but rather focuses on the story of the development of a few oxidizing bioreagents that, in a relatively short time, metamorphosed from exotic microorganisms and 'difficult to handle' enzymes into useful reagents for organic chemists.

2. A short history of the development of the prototype 'designer microorganisms'

While in the 1990s the use of lipase-catalyzed hydrolysis and esterification reactions was rapidly expanding, not least because many of the enzymes were available commercially, oxidations and reductions were lagging behind. Various oxidation reactions involving enzymes (e.g., monooxygenases, dioxygenases, oxidoreductases) requiring cofactors tended to be undertaken only in specialized laboratories, since the transformations utilized organisms, which required microbiological expertise to be grown and harvested before use.[7] Reduction of carbonyl compounds by *Saccharomyces cerevisiae*, baker's yeast, was a notable exception, mainly because of the tradition, generality, availability, and simplicity of use that allowed the reactions to be carried out in any laboratory. Yeast-catalyzed reductions have been performed by chemist for almost a century and are applicable to a wide range of aldehydes and ketone.[8] Inexpensive baker's yeast is readily available throughout the world from baking or brewing industries and the experimental protocols are very simple: dried yeast cells are rehydrated in the presence of sucrose (or glucose), the substrate is added, allowed to react, and the alcohol product is recovered by solvent extraction.[9] In sum, baker's yeast has been a chemist-friendly bioreagent.

As a result of contamination with petroleum products various microorganisms developed strategies to metabolize xenophobic compounds such as the alicyclic and aromatic hydrocarbons that are important components of petroleum. One of the key reactions in the degradation pathways of these compounds is Baeyer–Villiger oxidation involving C–C bond cleavage and insertion of an oxygen atom next to the alicyclic or aromatic keto group. Baeyer–Villiger oxidation[10] catalyzing enzymes (Baeyer-Villigerases, BVMOs) have been found in a variety of bacterial and fungal species.[11–13] Initially investigated for their involvement in metabolic degradation pathways,[11] they became the focus of more intense studies when, in 1976, Trudgill reported the isolation and characterization of cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIB 9871. Trudgill and co-workers remarked that CHMO, in addition to cyclohexanone and cyclopentanone, was able to catalyze oxidation of a variety of cyclic ketones to the corresponding lactones.[14] Although enantiomeric purity and absolute stereochemistry of the lactonic products were not reported at that time,[14] the importance of CHMO enzymatic activity attracted attention of several groups. Its mechanism[15] and stereochemistry[16] were investigated and its gene was cloned and sequenced.[17]

The initial studies already hinted that CHMO has potential as a synthetic reagent.[14,18,20] However, it was Taschner and Black in 1988,[18a] and Furstoss and co-workers 1989,[19a] who demonstrated that CHMO not only accepts a structural variety of cyclic ketones but also that it performs such oxidations with a very high enantioselectivity.[18,19] By the early 1990s groups in the US and Europe joined in exploration of selectivity and synthetic applications of CHMO[12] and related monooxygenases from other microorganisms.[21] The results

were exciting; CHMO, in particular, was found to be adept at converting a large variety of ketones (over hundred different structures) into highly optically pure lactones.[12]

In organic synthesis, the Baeyer–Villiger reaction is a major tool for C–C bond cleavage and oxygen insertion; unfortunately the reagents, such as *m*-chloroperbenzoic acid and trifluoroperacetic, used in these transformations are toxic, explosive, and give racemic products.[22] Yet, despite its demonstrated potential cyclohexanone monooxygenase, unlike lipases, was not entering into the repertoire of asymmetric reagents of organic chemists. Could the reluctance of the organic community to embrace this environmentally superior, efficient, and enantioselective bioreagent, be due to lack of accessibility?

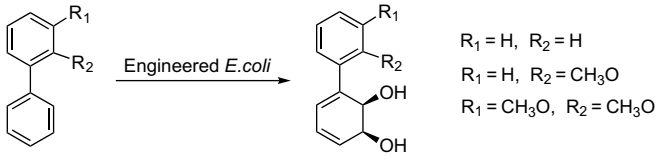
In the early 1990s biotransformations were performed either with isolated enzymes or with whole-cell native organisms. Isolation and purification of an enzyme requires equipment and expertise, particularly when an enzyme is not very stable, which is the case of CHMO. Furthermore, biotransformations with CHMO, an NADPH-dependent enzyme, necessitate cumbersome and expensive cofactor recycling. The whole-cell transformations dispense with enzyme isolation and provide the 'built-in' NADPH recycling system, but they present problems of their own. The use of *Acinetobacter* sp. is complicated by its pathogenicity,[23] the possibility of over metabolism of the lactones,[24] a relatively low production of enzyme in the cell, and the need to grow the organism on cyclohexanol that makes for difficult product isolation. Finally, cell growth and enzyme isolation require biochemical expertise and specialized equipment not found in a typical organic laboratory. In our opinion that was enough to discourage most organic chemists.

With the advent of molecular engineering it became possible to produce large quantities of desired proteins in new hosts such as bacterium *Escherichia coli* or fungus *S. cerevisiae* (overexpression systems).[25] Since these organisms do not exhibit BVMO activity they are suitable hosts for recombinant BVMO engineering.

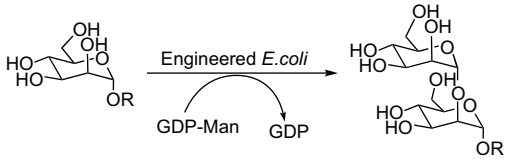
The 'designer yeast', a heterologous gene expression of cyclohexanone monooxygenase in baker's yeast was devised to appeal to non-specialists. It combined the chemistry of the isolated CHMO with the facility of baker's yeast-catalyzed reactions.[26] The 'designer yeast' turned out to be success; non-pathogenic, efficient (generating more CHMO than *Acinetobacter*), it can be prepared in bulk, frozen and stored at −80 °C. Moreover, BV/yeast-catalyzed reactions are as simple as the familiar baker's yeast reductions. Engineered yeast performed a variety of Baeyer–Villiger oxidations with high selectivity, excellent yields, and minimal side reactions.[12] The initial screenings showed that 2-, 3- and 4-substituted cyclohexanones, 2-, 3-substituted cyclopentanones as well as several sulfides were suitable substrates for the recombinant yeast. 4-Substituted cyclohexanones were oxidized in excellent yields to highly enantiopure (*S*)-lactones[26] (Scheme 1). Biotransformations of several 2-substituted cyclohexanones resulted in efficient kinetic resolution[27] while transformations of 3-substituted cyclohexanones were regio- and enantioselective.[28] Similarly, encouraging results were obtained in biooxidations of 2- and 3-substituted cyclopentanones[29] and sulfides.[30]

While these results have shown that the engineered yeast strains can carry out asymmetric thioether oxidations, side reactions catalyzed by resident yeast enzymes were occasionally a problem. Engineering a strain of *E. coli* that overexpresses the same enzyme was a possible solution. Since the original *E. coli* expression for CHMO generated only a small quantity of the protein, comparable to that produced by native *Acinetobacter* cells,[17] an updated overexpression[30] of CHMO in *E. coli* was bioengineered.[31] The *E. coli*/*CHMO*, driven by a strong T7 promoter generates CHMO at 20% of total cellular protein,[30] is non-toxic, can be stored at −80 °C for months and is as easy to handle as the designer yeast but produces less biomass.[31] In view of these advantages, *E. coli*/*CHMO* has been used preferentially in the majority of biotransformations.[32] Within a few years other overexpression systems employing slightly different *E. coli* hosts and/or promoters were described [33] and the *E. coli*/*BVMO* overexpression systems became favorite Baeyer–Villiger bioreagents of many research groups. In the years that followed, *E. coli* overexpression systems of several cyclohexanone monooxygenases and numerous other Baeyer-Villigerases were constructed and were used to explore substrate profiles of these bioreagents.[34,35]

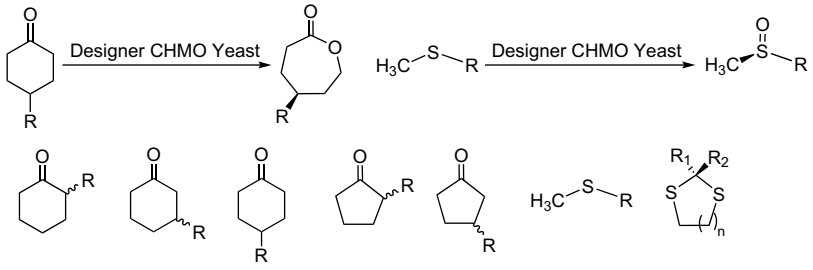
The success of the 'designer' yeast and *E. coli* as bioreagent for Baeyer–Villiger oxidations coincided with the development of other 'microbial bioreagents.' For example, Hudlicky and co-workers[36] used *E. coli* JM109 (pDTG601), a recombinant microorganism that overexpresses the enzyme toluene dioxygenase from *Pseudomonas* strains,[37] to prepare on a multi-gram scale enantiomerically pure 3-(2-methoxyphenyl)-(1*S*,2*R*)-3,5-cyclohexadiene-l,2-diol and 3-(2,3-dimethoxyphenyl)-(1*S*,2*R*)-3,5-cyclohexadiene-l,2-diol as the only reaction products (Scheme 2), while Wang and co-workers engineered *E. coli* expressing yeast α-l,2-mannosyl transferase for a series of mannosylation reactions[38] (Scheme 3).



Scheme 2.



Scheme 3.



Scheme 1.

In the past, metabolic engineering focused principally on the overproduction of natural metabolites and production of novel metabolites.[25,39] The 1990s brought with them another application: engineered microbes specifically designed and constructed as reagents for organic synthesis.[25] A decade later the recombinant host organisms overexpressing oxygenases and reductases and other synthetically useful enzymes from a variety of microorganisms are routinely bioengineered, used as bireagents, and extensively studied for their applications in synthesis in organic laboratories and industry. It is also important to remember that isolated enzymes destined for research, as well as many commercially available enzymes, are produced by the same recombinant systems.

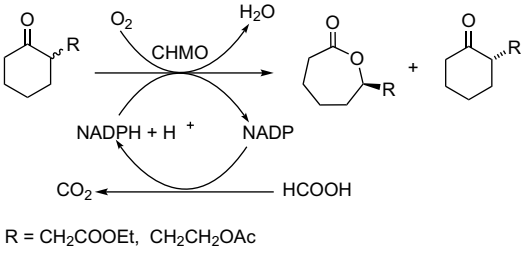
3. Isolated enzymes versus designer organisms

Development of efficient *E. coli* overexpression systems for CHMO from *Acinetobacter* sp. NCIB 9871 simplified the whole-cell catalyzed Baeyer–Villiger oxidations of many ketones and sulfides.[12,40] Furthermore, because the engineered cells express more of the target protein[41] it also provided better access to the isolated enzyme. This, coupled with a relatively easy purification of the recombinant enzyme that was accomplished by incorporation of a His_6-tag at the C-terminus of CHMO,[33b] facilitated mechanistic studies[42] and promoted development of biotransformations with the isolated enzymes.

The principal difficulties encountered in reactions catalyzed with isolated oxidoreductases are associated with enzyme stability and cofactor regeneration. Cofactor regeneration is necessary to reduce the cost. It also prevents problems of product inhibition by the cofactor, facilitates product isolation, and can drive thermodynamically unfavorable transformation by coupling it with favorable regeneration reactions.[43] The combination of immobilization of an enzyme with more efficient and less expensive methods for regeneration of NADPH cofactor simplified biotransformations with Baeyer-Villigerases and other enzymes requiring cofactor.[43] The following examples illustrate this point.

3.1. NADPH recycling strategies

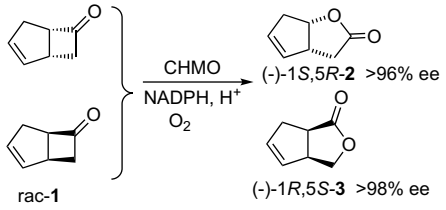
The $NADP^+$ dependent formate dehydrogenase (FDH) from *Pseudomonas* sp. 101 was engineered by multiple site-directed mutagenesis to accept formate as substrate. It allowed for an efficient and inexpensive method for the regeneration of NADPH.[44,45] The new regeneration system was coupled with the isolated cyclohexanone monooxygenase and tested in the oxidation of prochiral and racemic cyclohexanones.[44,46] The experimental set-up required gentle but efficient aeration since the standard methods of oxygen delivery by bubbling air, or by intensive stirring, deactivated the enzyme. The development of a bubble-free aeration allowed conversion of substituted cyclohexanones to the corresponding ε-lactones to be performed on a preparative scale[44,46] (Scheme 4).



Scheme 4.

3.2. Enzyme immobilization

Zambianchi and co-workers investigated Baeyer–Villiger and sulfide oxidations with isolated cyclohexanone monooxygenase.[47] A *partially purified* enzyme from the 'designer *E. coli*' overexpression system was used,[33a] while 2-propanol coupled with alcohol dehydrogenase from *Thermoanaerobium brocki* was employed in NADPH regeneration. Immobilization on Eupergit C stabilized the enzyme (increasing half-life from 1 to approximately 2.5 days) and allowed reuse of the catalyst up to 16 times with complete substrate (5 g/L) conversion at each cycle.[47] In the case of bicyclo[3.2.0]hept-2-en-6-one **1**, continuous substrate feeding shortened reaction times and yielded both lactone products with high optical purity (enantiomeric excess ≥96%), which was not the case when all of the substrate was added in a single batch (Scheme 5).



Scheme 5.

In summary, the NADPH recycling system employing CHMO/engineered FDH[44] was shown to be effective in Baeyer–Villiger oxidations of several substituted cyclohexanones.[44,45] This system, however, is of a relatively low efficiency[47] and, although it is often preferred over glucose-6-phosphate dehydrogenase that uses an expensive glucose-6-phosphate substrate, NADPH regeneration is still best carried out in whole cells.[43]

As for enzyme immobilization there are several advantages to attach enzymes to a solid support. When biotransformation is carried out with an enzyme, reactant and product in solution, the separation of product is difficult and the enzyme is destroyed. Immobilized enzyme, on the other hand, can be rapidly removed and can be re-used, and the product is not contaminated with the enzyme. Furthermore, enzymes are usually stabilized upon attaching to solid support and in some cases give better conversions.[48] That, however, depends on an enzyme, supporting material,[49] and reaction conditions, and requires much experimentation by experts.

Finally, comparative study of CHMO isolated from *Acinetobacter* with that overexpressed in *E. coli* (TOP10 pQR239) showed them to be practically identical. The slight difference in pH and thermal stability was ascribed to the differences in trace impurities with proteolytic enzymes present in *E. coli* host.[50] Thus, although the progress in the use of isolated enzymes is impressive and finds extensive industrial applications, at the present time biotransformations with intact cells of 'designer' bioreagents are more accessible and more chemist friendly than the biotransformations with isolated enzymes.

4. Currently available designer organisms and what they can do for a chemist

4.1. Cyclohexanone monooxygenase ($CHMO_{Acineto}$)

CHMO from *Acinetobacter* sp. NCIB 9871 is classified as a type I BVMO; this means that it contains flavine adenine dinucleotide (FAD) as a cofactor and uses NADPH as source of electrons.[13,51] It is by far the most extensively studied Baeyer–Villiger

monooxygenase. The investigation of CHMO's mechanism, originally published in 1982[15a] was refined in 2001 using double-mixing stop-flow techniques;[42] the accepted catalytic cycle may be summarized as shown in Figure 1.[42] In the resting enzyme, non-covalently bound FAD is in its oxidized form and has to be reduced by NADPH to the reduced enzyme–$NADP^+$ complex, which is then oxidized by molecular oxygen to the **4a**-flavin peroxide. The reactive intermediate, **4a**-flavin peroxy anion, acts as a nucleophile toward ketones (and boronic acids)[15b,52] but in its protonated form it can act as an electrophile vis-a-vis heteroatoms such as S or N (also Se, P).[53] In the presence of a ketone, nucleophilic attack on the carbonyl group by **4a**-flavin peroxide leads to the formation of Criegee intermediate (just as in peracid-catalyzed chemical Baeyer–Villiger reactions), which rearranges to lactone and flavin hydroxide. The elimination of water followed by release of the product and $NADP^+$ complete the cycle. The released $NADP^+$ must be reduced (outside the enzyme) to return as NADPH for the next turnover. That is why in the isolated enzyme-catalyzed oxidations a continuous supply of very expensive NADPH is required. Alternatively, an efficient recycling system for NADPH must be provided.

Thus, in biotransformations **4a**-hydroperoxyflavin is an equivalent of a peracid used in chemical Baeyer–Villiger reactions; but in the world of an enzyme the oxidizing agent is firmly fixed within the chiral environment of the active site. It is that chiral environment that confers on an enzyme its selectivity and it is such chiral or asymmetric environment that chemists seek to create when designing enantioselective catalysts. The exceptional characteristic of CHMO is not that it is highly enantioselective (most enzymes are), or that it accepts a wide range of substrates, but that it is highly enantioselective toward a great variety of substrates.

4.2. CHMO's substrate base

CHMO is an amazing enzyme and the 'designer yeast and *E. coli*' CHMO overexpression systems are true 'bioreagents'. Their ability to oxidize, with high chemo-, regio-, and enantioselectivity, hundreds of structurally diverse compounds are unparalleled. The comprehensive list of ketonic substrates was first published in 1998[12] and has been constantly growing.[34,36] It includes cyclic, bicyclic, tricyclic, and heterocyclic ketones with a variety of substituents and substitution patterns.[12,34,36] CHMO oxidizing capacity does not stop at the Baeyer–Villiger reactions, it also oxidizes enantioselectively a range of sulfides,[53–55] dithienes, dithiolanes,[56] and converts tertiary, secondary, and hydroxylamines to *N*-oxides, hydroxylamines, and nitrones, respectively.[57] Finally, the first asymmetric epoxidation has been reported recently.[58] This epoxidation is highly enantioselective but limited to dimethyl and diethyl vinyl phosphate (phenyl vinyl sulfoxide was oxidized to sulfone). The structural features of the compounds accepted as substrates by CHMO are shown in Figure 2.

4.3. CHMO's enantioselectivity

CHMO converts prochiral ketones into the corresponding enantiopure or highly enantioenriched lactones (desymmetrization) and kinetically resolves racemic mixtures. For example, the six-membered heterocyclic ketone **4** is converted to the lactone **5** in good yield and with very high enantiomeric excess (79% yield; 99% ee), (Scheme 6).[59] The kinetic resolution of racemic mixtures on the other hand leads to the formation of enantiopure lactone in maximum 50% yield at best, leaving behind the unreacting enantiomer—the remaining 50% of the original mixture. The biooxidation

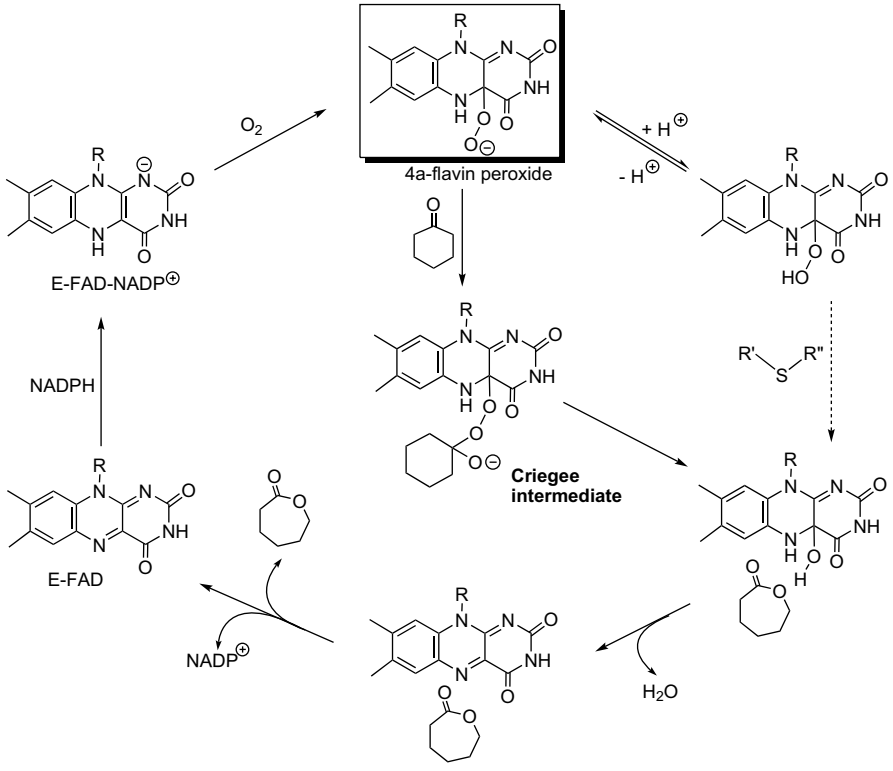


Figure 1. Mechanism of cyclohexanone monooxygenase. In the resting enzyme protein bound-FAD is in its oxidized form (E-FAD). It has to be reduced by NADPH and in the process the reduced enzyme–$NADP^+$ complex is formed, which is oxidized by molecular oxygen to **4a**-flavin peroxide. The reactive intermediate, **4a**-flavin peroxide, acts as a nucleophile toward ketones, but in its protonated form it can act as an electrophile vis-a-vis heteroatoms such as S. In the presence of a ketone, nucleophilic attack on the carbonyl group by **4a**-flavin peroxide leads to the formation of Criegee intermediate (just as in peracid-catalyzed chemical Baeyer–Villiger reactions), which rearranges to give lactone and flavin hydroxide. The elimination of water followed by release of the product and $NADP^+$ complete the cycle.

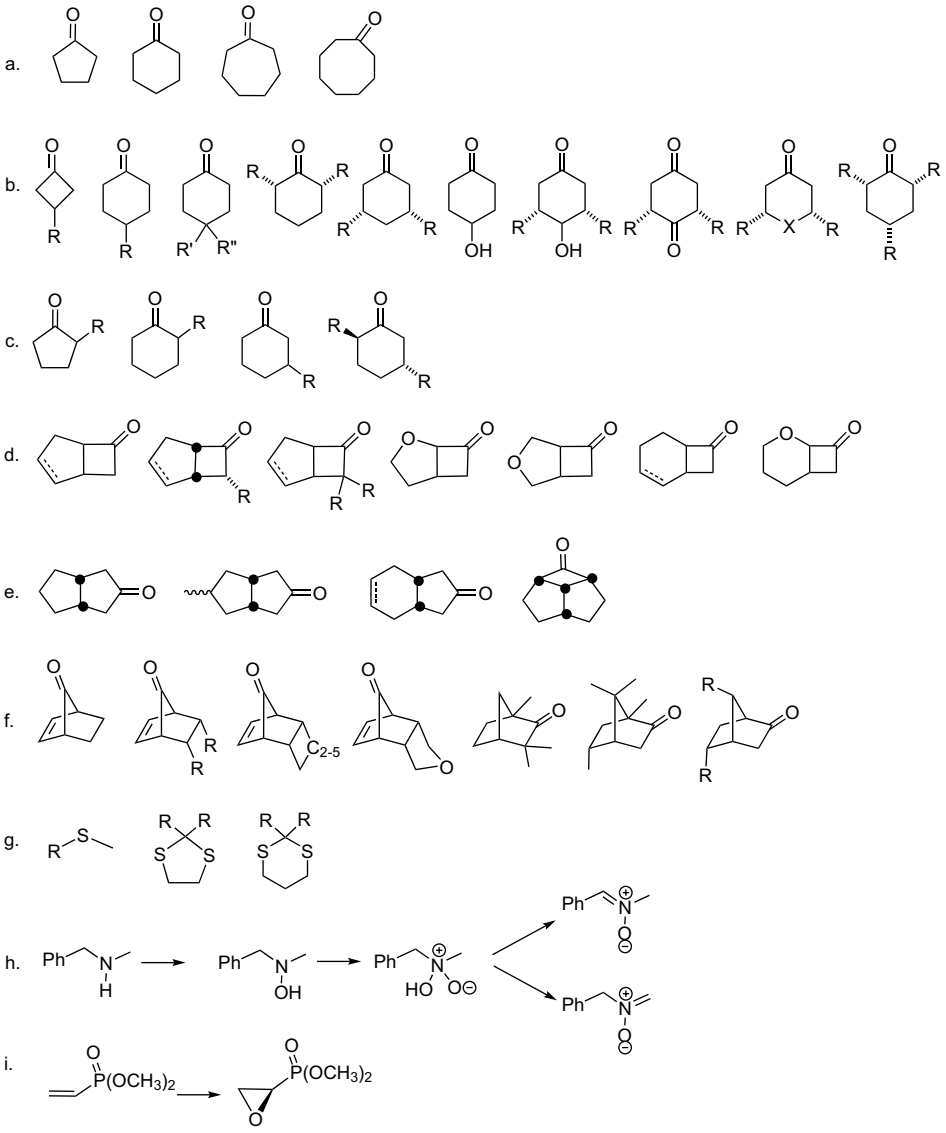
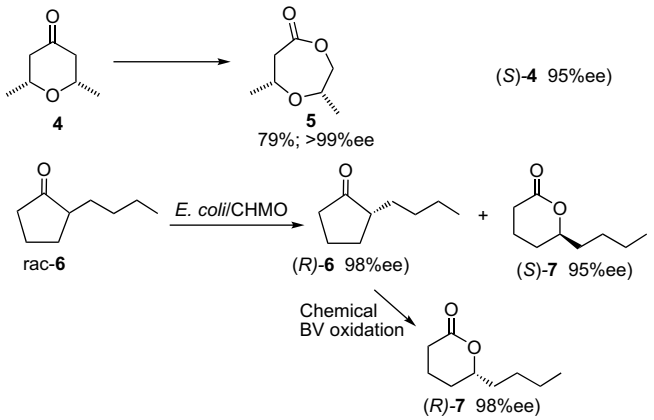


Figure 2. Representative substrates of cyclohexanone monooxygenase showing the ring systems and substitution patterns accepted by the CHMO from *Acinetobacter* sp. A variety of R groups has been investigated. These include aliphatic, branched aliphatic, aromatic, substituted aromatic, aliphatic/aromatic, and heteroatom containing chains. The details of the conversions of substrates a, b, d, f can be found in Refs. 12 and 34 and for structures c and e in Ref. 34.

of racemic 2-propylcyclopentanone **6** illustrates such kinetic resolution. In this case, *E. coli*/CHMO-catalyzed reaction was quenched at 49% conversion and the lactone **7** (*S* 95% ee) and ketone (*R*)-**6** (98% ee) were obtained both in 47% yield.[60] Because non-enzymatic Baeyer–Villiger oxidations proceed with retention of configuration, ketone (*R*)-**3** (98% ee) can be chemically oxidized to (*R*)-**7**(98% ee) therefore providing access to both antipodes of the lactone.



Scheme 6.

When only a specific enantiomer is required, conventional kinetic resolution is less attractive because the maximum yield is limited to 50%. In some cases, this problem can be solved if a dynamic kinetic resolution can be set up. Dynamic kinetic resolution (DKR) is a method that combines enzymatic kinetic resolution with the in situ racemization of the substrate.[61] Over the years, the enzyme-promoted kinetic resolution of racemates has been extensively explored and the number of reported chemoenzymatic DKR has been growing.[4a,b,61] The first example of a DKR performed by recombinant *E. coli*/CHMO[62] has been reported recently and is shown in Figure 3.

Base-catalyzed substrate racemization at pH 8.5 was adequately rapid for dynamic resolution. The shortcoming of this procedure was that substrate concentration had to be very low. To improve the process the whole-cell-catalyzed Baeyer–Villiger oxidation was combined with an ion exchange resin-catalyzed in situ

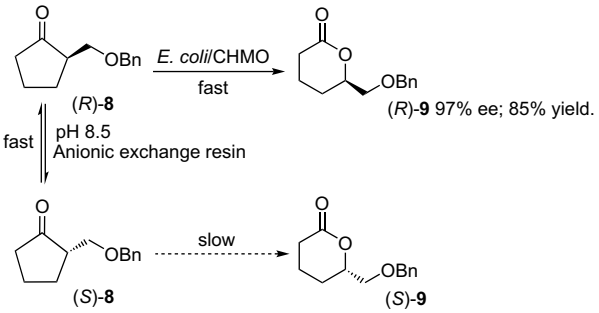


Figure 3. Dynamic kinetic resolution (DKR) of 2-benzyloxymethylcyclopentanone using recombinant *E. coli*/CHMO. Here, due to relative acidity of the α-proton, spontaneous racemization via a keto/enol tautomerism occurs at pH 9. This racemization is as fast, or faster, than the oxidation of the 'faster' (*R*)-**8** enantiomer allowing for successful DKR.[62b]

racemization of the substrate. The new protocol employing weakly basic Lewatit MP62 resin allowed for significant improvement in substrate concentration and gave lactone (*R*)-**9** (97% ee) in 84% isolated yield.[62b]

4.4. CHMO's regioselectivity

In Baeyer–Villiger reactions the tetrahedral Criegee intermediate rearranges to lactone with preferential migration of the more nucleophilic carbon center. Thus, in Baeyer–Villiger oxidations of 2-substituted cyclohexanones the more substituted α-carbon atom migrates in both chemical and enzymatic reactions. Chemical oxidations of 3-substituted cyclic ketones give two enantiomeric pairs of regiomeric lactones since there is no electronic preference for one of the C–C bond to migrate. Enzymatic reactions, however, can be highly regioselective; for example, in the yeast/CHMO mediated oxidation of racemic 3-methylcyclohexanone **10**, the two enantiomers were converted to two regiomers **11** and **12** (Fig. 4). The divergent behavior of enantiomers was earlier observed in CHMO oxidations of racemic bicyclo[3,2,0]hept-2-en-6-one **1** (Fig. 4) and several other fused bicyclobutanones by Alphand and co-workers.[19a,63]

For stereoelectronic reasons the rearrangement of the Criegee intermediate occurs through the migration of the alkyl group antiperiplanar to the O–O bond (Fig. 5). When the conformation of the O–O bond is not regulated, the more substituted alkyl group (the more nucleophilic group) migrates preferentially; however, when the conformation of the intermediate imposes placement of the *less* substituted group antiperiplanar to the O–O bond, it is that group that will migrate preferentially. In other words, stereoelectronic effect is more important in stabilizing the transition state than the preferential migration of the more nucleophilic carbon

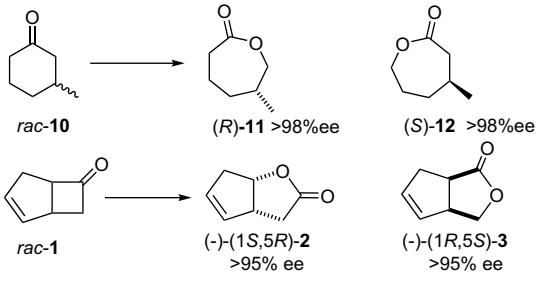


Figure 4. In regiodivergent Baeyer–Villiger oxidations one enantiomer of the ketonic substrate is selectively oxidized to one regioisomer and its antipode is converted to the other regioisomer.

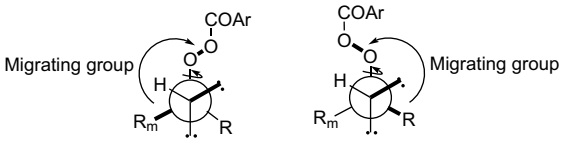
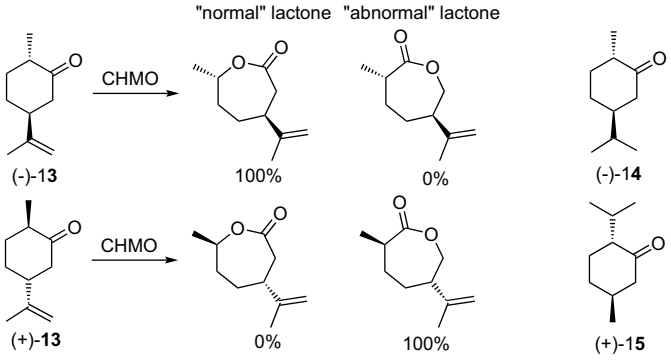


Figure 5. The primary stereoelectronic effects operating in the rearrangement of Criegee intermediate: the migrating group is antiperiplanar to the O–O bond. When either group can migrate (free rotation), the more substituted (more nucleophilic) carbon will migrate preferentially. In an environment imposing only one possible conformation, the antiperiplanar group migrates.

center. This happens in a variety of enzymatic reactions. The active site of an enzyme, because of its chiral environment, frequently imposes restrictions that allow only a single conformation of the Criegee intermediate. In that case, the group antiperiplanar to O–O migrates, regardless of it nucleophilicity. Or, one enantiomer can assume the configuration where the more substituted group is antiperiplanar and migrates (the normal product), while the other enantiomer is forced into a configuration that leads to the migration of the less substituted carbon only (the abnormal product). Recently, Watanabe and co-workers reported the first regiodivergent chemocatalytic Baeyer–Villiger oxidation catalyzed by Zr[bis(salicylidene)ethylene-diaminato](salen) complex.[64]

There are other cases of regiodivergent CHMO oxidations. Sometimes both enantiomers of a substrate ketone are accepted. For example, oxidations of pure enantiomers of *trans*-dihydrocarvone **13** and carvomenthone **14** give 'normal' and 'abnormal' lactones depending on the absolute configuration of the ketone precursor. Here, enantiomeric purities (determined using chiral GC analysis) were higher than 98%; conversion was excellent for **13** but only modest for the compound **14**.[65] In some cases, only one enantiomer is accepted and is converted to a single product; for example, only (+) menthone **15** is oxidized and it is oxidized to 'normal' lactone with high ee value, (Scheme 7).[65]



Scheme 7.

4.5. Other Baeyer-Villigerases

Although it has been known for decades that many bacteria and fungi produce Baeyer–Villiger enzymes, by the early 1990s only a few of these enzymes were isolated, purified and characterized.[12] These included cyclohexanone monooxygenase from *Acinetobacter* and from *Nocardia globerula* CL1,[14] cyclopentanone oxygenase from *Pseudomonas* (later identified as *Comamonas*) NCIB 9872[66] and three Baeyer–Villiger oxygenases involved in metabolism of camphor from *Pseudomonas putida* C1.[67] The construction of recombinant overexpression systems for CHMO from *Acinetobacter* in baker's yeast (*S. cerevisiae*) and in *E. coli*, and their success in regio- and enantioselective oxidation encouraged search for other BVMOs.

Within a few years several new BVMOs were overexpressed in *E. coli*. They included several cyclohexanone monooxygenases,[68–70] a steroid monooxygenase (SMO) from *Rhodococcus rhodochrous*[71,72] and cyclodecanone monoxygenase (CDMO), which was the first characterized enzyme to catalyze Baeyer–Villiger oxidation of large cyclic compounds.[73] In early 2000, overexpression systems in *E. coli* were engineered for 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB[74] and for cyclopentanone monooxygenase (CPMO) from *Comamonas* sp. NCIMB 9872[75] (Table 1).

Extensive studies of substrate specificities, enantio- and regioselectivities of the new BVMOs followed and many results have now been reported and reviewed.[36,51] The availability of several new recombinant in *E. coli*/BVMO bioreagents for Baeyer–Villiger oxidations allowed for a collective substrate base significantly larger than that of CHMO from *Acinetobacter* sp. NCIB 9871 ($CHMO_{Acineto1}$) alone. Furthermore, enantio- and regiodivergent enzymes for a large pool of substrates were identified, making access to the desired enantio- and regiopure lactones in excellent yields a reality.

4.5.1. Cyclohexanone monooxygenases (CHMOs)

A comparative study of substrate acceptance and enantioselectivity of eight new Baeyer–Villiger monooxygenases toward a series of 2-, 3-, and 4-substituted cyclohexanones showed them to have overlapping substrate bases and similar enantioselectivities.[76] The new CHMOs showed predominantly high (*S*)-selectivity toward 4-alkyl substituted cyclohexanones and a few excellent matches between a new monooxygenase and a specific substrate were identified. Thus, with the present collection of CHMOs it is possible to convert all 4-alkyl cyclohexanones tested to the corresponding (*S*)-lactones in better than 98% enantiomeric excess. In a few cases where (*R*)-lactones were the preferred product, the enantiomeric purity was very low.[76] All CHMOs studied favor the conversion of (*S*) 2-alkyl cyclohexanones, but only the original CHMO ($CHMO_{Acineto1}$) and the CHMO from *Rhodococcus* SC1 ($CHMO_{Rhodo}$) give lactones with high *E* values;[77] the selectivities of other CHMOs are low. No regiodivergence was observed in the oxidations of 3-alkyl cyclohexanones; none of the CHMOs in the study produced the distal lactones (structure **12** in Fig. 4). At best, lactone **12** was formed in a 1:1 ratio with proximal lactone **11**, more often they were the minor component in the product mixture. An interesting observation was made in the biotransformation of 3-*n*-butylcyclohexanone **10** (R=*n*-Bu). Here a pair of enzymes from *Brevibacterium* sp. was enantiodivergent, that is $CHMO_{Brevi1}$ gave (*R*)-lactone while $CHMO_{Brevi2}$ gave (*S*)-lactone.[76] In another study, biotransormations carried out with recombinant *E. coli* expressing the same two enzymes confirmed their enantiodivergent behavior vis-a-vis several structurally diverse mesomeric ketones.[78]

4.5.2. Cyclopentanone monooxygenase ($CPMO_{Coma}$)

Cyclopentanone monooxygenase from *Pseudonomas sp*. NCIMB 9872, recently reclassified as belonging to genus *Comamonas*,[75] was reported by Trudgill and Griffin[66] at the same time as its more famous cousin $CHMO_{Acineto1}$.[14] In early years it did not receive much attention in the investigation of Baeyer–Villiger oxidations of unnatural substrates. The availability of recombinant *E. coli*/$CPMO_{Coma}$[75] reopened interest in this enzyme. The initial experiments showed CPMO-catalyzed reactions to be fast and efficient, and although the selectivity was frequently low, there were signs that the substrate base might be broader than that of CHMO. As a result CPMO was found adequately different from CHMO to merit further development as an alternative bioreagent.[79] Indications that CPMO may transform aromatic[75] and conjugated ketones[80] that are not accepted by CHMO, further promoted interest in this enzyme (Fig. 6a and b). The comparative studies of two bioreagents (CHMO vs CPMO) showed that, although in the oxidations of many cyclopentanone and cyclohexanone substrates the selectivities of the two enzymes were frequently the same (with CHMO being generally more enantioselective), several examples of enantio- and regiodivergence were remarked.[75,79,81] A few enantiodivergent transformations of prochiral 4-substituted cyclohexanones by the two enzymes[81] are shown in Figure 7. As the interest in BVMOs increased and screening studies multiply, other examples of differences in substrate specificity, enantiodivergence, and regiodivergence between $CHMO_{Acineto}$ and $CPMO_{Coma}$ appeared in the literature.[82–85]

4.6. CHMOs versus CPMO: relationship and biocatalytic properties

A comparison of enantio- and regioselectivity of several (eight) CHMOs and $CPMO_{Coma}$ against a series of prochiral ketones with different structural characteristics (Fig. 8) showed that $CPMO_{Coma}$ and $CHMO_{Brevi2}$ produced consistently lactonic products antipodal to the remaining six CHMOs.[65,86,87] These results were in accord with previously observed enantiodivergence between $CHMO_{Brevi1}$ and $CHMO_{Brevi2}$,[76] and enantiodivergence of $CHMO_{Acineto}$ and $CPMO_{Coma}$.[75,79,81] Distinct selectivity of the two enzymes ($CPMO_{Coma}$ and $CHMO_{Brevi2}$) was manifested also in Baeyer–Villiger oxidation of fused ketone **10** (Fig. 4) where the 'normal' lactone **11** was formed as a major product (98%) while the remaining CHMOs

Table 1
The list of Baeyer–Villiger monooxygenases discussed in this report

Organism	Monooxygenases	Designation	Ref.
Acinetobacter sp. NCIMB 9871	Cyclohexanone	$CHMO_{Acineto1}$	14
Acinetobacter SE 19	Cyclohexanone	$CHMO_{Acineto2}$	69,76
Arthrobacter BP2	Cyclohexanone	$CHMO_{Arthro}$	70
Brachymonas petroleovorans	Cyclohexanone	$CHMO_{Brachy}$	68b
Brevibacterium HCU	Cyclohexanone	$CHMO_{Brevi1}$	68a
Brevibacterium HCU	Cyclohexanone	$CHMO_{Brevi2}$	68a
Rhodococcus Phi1	Cyclohexanone	$CHMO_{Rhodo1}$	70
Rhodococcus Phi2	Cyclohexanone	$CHMO_{Rhodo2}$	70
Rhodococcus SC1	Cyclododecanone	$CDMO_{Rhodo}$	73
P. fluorescens ACB	Hydroxyacetophenone	$HAPMO_{Pseudo}$	74
Comamonas NCIMB 9872	Cyclopentanone	$CPMO_{Coma}$	75
R. rhodochrous IFO 3338	Steroid	SMO_{Rhodo}	72
P. lilacinum	Steroid		96
Penicillium citreoviride A.C.C.C.0402	Steroid		97
Xanthobacter sp. ZL5	Cyclohexanone	$CHMO_{Xantho}$	88
T. fusca	Phenylacetone	PAMO	102
Pseudomonas sp. strain HI-70	Cyclopentadecanone	CPDMO	104

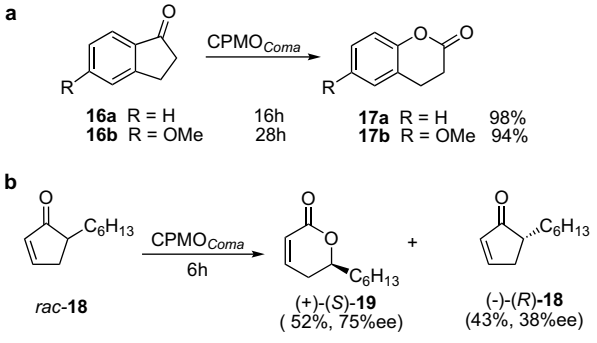


Figure 6. (a) 2,3-Dihydroinden-1-one **16a** and 2,3-dihydro-5-methoxyinden-1-one **16b** are excellent substrates for CPMO but are not accepted by CHMO.[75] (b) Biotransformation of 5-hexylcyclopent-2-enone by isolated CPMO enzyme gives the α,β-unsaturated δ-hexyl valerolactone with modest enantioselectivity;[80] CHMO does not transform conjugated ketones.

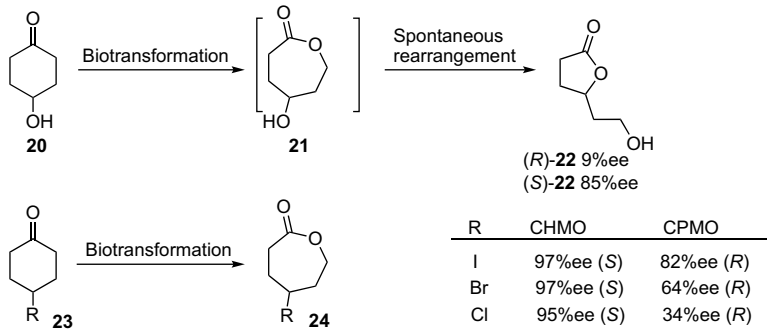


Figure 7. The enantiodivergence of CHMO and CPMO can be observed in the oxidations of 4-hydroxy- and 4-halocyclohexanones.[81]

gave both 'normal' and 'abnormal' lactones in approximately the same amounts.[86b]

To rationalize the similarity between $CHMO_{Brevi2}$ and CPMO and their divergence from other CHMOs, experimental selectivity results were confronted with the sequence data for genes and proteins of all the participating BVMOs. A trend in enzyme similarity at the amino acid level allowed grouping $CHMO_{Brevi2}$ and CPMO in one branch with the remaining CHMOs belonging to another cluster (Fig. 9).[86a] The borderline position of $CHMO_{Brevi1}$ often reflected in its stereopreference and substrate acceptance supports the proposed protein sequence/biocatalytic properties relationship.[86a]

It should be stressed, however, that the enantiopreference divergence of $CHMO_{Brevi}$ and CPMO from that of the other CHMOs is not a rule. For example, in the oxidations of 3-substituted cyclobutanones only two out of ten substrates studied were consistent with CHMO-type, CPMO-type classification.[86d] These results underscore the empirical character of the CHMO-, CPMO-type classification.

A recent addition to the cyclohexanone monooxygenase family, a CHMO from *Xanthobacter* sp. ZL5,[88] screened against the same set of substrates was found to be closely related to the CHMO-type cluster, particularly $CHMO_{Acineto}$, on the basis of its substrate acceptance and selectivity. $CHMO_{Xantho}$ ability to oxidize sterically hindered bi- and tricyclic compounds is of special interest because it is generally highly enantioselective and, in a number of cases it is the only enzyme known that can accept and transform a particular substrate or provide the antipodal lactone (Fig. 10).[87]

4.7. New BVMOs with synthetic potential

4.7.1. 4-Hydroxyacetophenone monooxygenase (HAPMO)

In addition to the metabolism of cyclic ketones the degradation of aryl ketones can also proceed via Baeyer–Villiger oxidation, and aromatic degradation pathways involving Baeyer–Villiger oxidation have been known since 1970s.[89] However, the first functional BVMO enzyme, hydroxyl-acetophenone monooxygenase (HAPMO) was isolated and purified from *P. fluorescens* ACB only in 1999.[90] Within two years, it was characterized, cloned, overexpressed in *E. coli*, and its substrate specificity was investigated.[74] HAPMO is a versatile enzyme with a broad substrate specificity. In addition to 4-hydroxyacetophenone **31**, its natural substrate,[91] HAPMO accepts a wide range of aryl ketones and a variety of other compounds including heteroaromatic ketones, aliphatic ketones, and sulfides, Figure 11.[74b]

HAPMO displays high catalytic activity for oxidation of aromatic and heteroaromatic ketones but only moderate acceptance of aliphatic ketones. In the transformation of acetophenones a substituent at the *para* position appears to be important. *p*-Hydroxy or *p*-amino substituted acetophenones are better substrates than acetophenones substituted with a hydrophobic or bulky group. An electron withdrawing group, such as fluorine in *para* positions lowers the enzyme's activity, while a 4-nitroacetophenone is not converted by HAPMO. The *meta* and *ortho* positions do not have significant effects on the kinetic parameters of the transformation. Modification of the aceto function influences both the affinity and the rate of catalysis. The best results are observed with acetophenones and propiophenones. The heteroaromatic and other

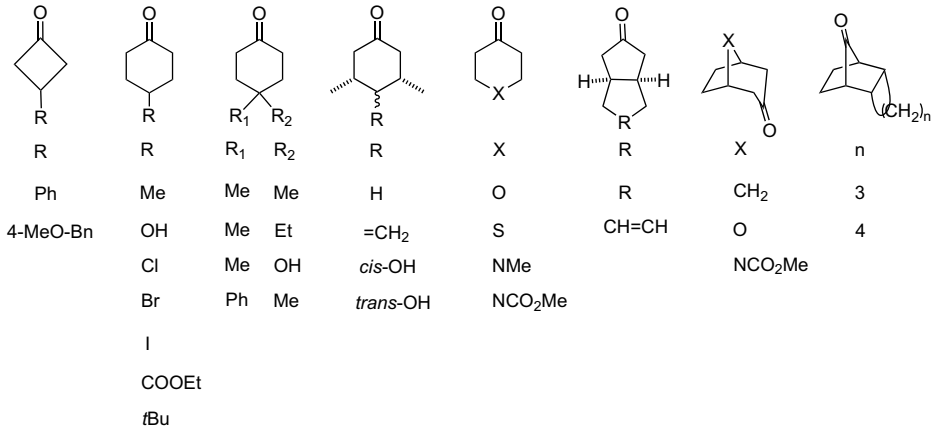


Figure 8. Biooxidation of structurally diverse prochiral ketones by seven CHMOs and $CPMO_{Coma}$ showed that all CHMOs with exception of $CHMO_{Brevi2}$ have the same enantiopreference (i.e., give lactones with the same configuration) although enantioselectivity (% ee) and the degree of conversion (substrate acceptance) varied significantly. Oxidations by $CHMO_{Brevi2}$ and CPMO produced lactones of opposite configuration than those obtained from transformations with other six CHMOs. The degree of conversion and enantiopurity of the $CHMO_{Brevi2}$ and CPMO products were quite similar.[86]

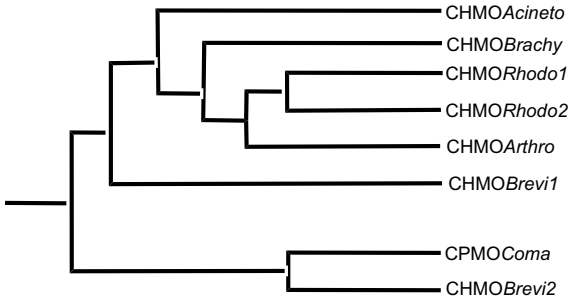


Figure 9. The relationship between $CHMO_{Brevi2}$ and CPMO (CPMO-type enzymes) and the other six CHMOs (CHMO-type enzymes).

compounds shown in Figure 11b are all converted by HAPMO in moderate to good yields. Oxidation of several sulfides studied leads to essentially enantiopure sulfoxides.[74b]

Bicyclic ketones (shown in Fig. 11) are converted in moderate yields to a mixture of 'normal' and 'abnormal' lactones, in most cases with low enantiomeric excess.[92] Similarly, HAPMO-catalyzed desymmetrization of 3-substituted cyclobutanones produced corresponding valerolactones in moderate yields and, with the exception of 3-phenyl-cyclobutanone, rather low ee values. An interesting aspect of the latter transformations is that HAPMO often provides an access to a lactone with the configuration that is not accessible through transformations with other BVMOs (Fig. 12). In conclusion, HAPMO adds a distinctly different substrate base to the collection of other BVMOs. In case of an overlap with the substrate bases of other monooxygenases HAPMOs stereopreference for desymmetrization as well as regiodivergence is distinct in a number of cases studied.

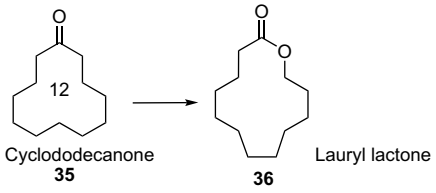
4.7.2. Steroid monooxygenase (SMO)

The earliest reports on the Baeyer–Villiger biooxidations came from studies of steroid metabolism in several fungi by a group at the Upjohn Company. Thus, in 1953 Peterson and co-workers reported conversion of progesterone to androstenedione, testololactone by *Penicillium*, *Aspergillus*, and *Gliocladium*.[93] Because of the important bioactivities of the testololactones,[94] progesterone metabolism in a variety of fungi was investigated in more detail and the metabolic pathway that includes two Baeyer–Villiger oxidations was established (Fig. 13).[95–97]

An overexpression system in *E. coli* constructed in 1999 for SMO from *R. rhodochrous* [72] allowed for 40-fold protein production compared to the parent organism. This made SMO a valuable potential bioreagent. It is quite possible that these enzymes, like other BVMOs, can convert compounds that are quite different from their natural substrates; however, substrate acceptance studies of SMOs have been restricted to compounds closely related to steroids involved in the metabolic pathways and a broader range of potential substrates has not been established thus far.

4.7.3. Cyclododecanone monooxygenase (CDMO)

The alicyclic hydrocarbons are important components of petroleum and many microorganisms metabolize them. The majority of these microbes isolated to date contain degradation pathways for small ring (C5 to C7) cyclic compounds and the substrate specificity of the monooxygenase enzymes involved is generally limited to small ring sizes. A strain that uses cyclododecane as the sole carbon source and is able to degrade large cyclic hydrocarbons was obtained from *Rhodococcus ruber* CD4 and the enzyme, cyclododecanone monooxygenase (CDMO), required for the oxidation of cycloketone to the corresponding lactone was isolated and purified.[98] The *E. coli* overexpression system for CDMO was engineered from the closely related microorganism *R. ruber* SC1. The recombinant *E. coli*/CDMO strain converts cyclododecanone **35** to the lauryl lactone **36** and oxidizes long-chain cyclic ketones (C11 to C15) to the corresponding lactones but is essentially inactive toward small-ring systems.[99] CDMO capacity to convert large ring system is an important addition to the monooxygenases based reagents and its full range of substrates needs to be explored.



4.7.4. Aliphatic ketones monooxygenase ($AKMO_{Pfluor}$)

A newest addition to the BVMO cluster is a BVMO from *P. fluorescens* DSM 50106.[100] This new, yet nameless, BVMO was cloned and expressed in *E. coli*. Screening experiments revealed its high specificity for aliphatic open-chain ketones. Despite the fact that it exhibits high sequence similarity (37%) to 4-hydroxyacetophenone monooxygenase (HAPMO), AKMO does not accept cycloketones or aromatic ketones but shows preference for short-chain alicyclic ketones. Thus, while decan-2-one and undecan-2-one were

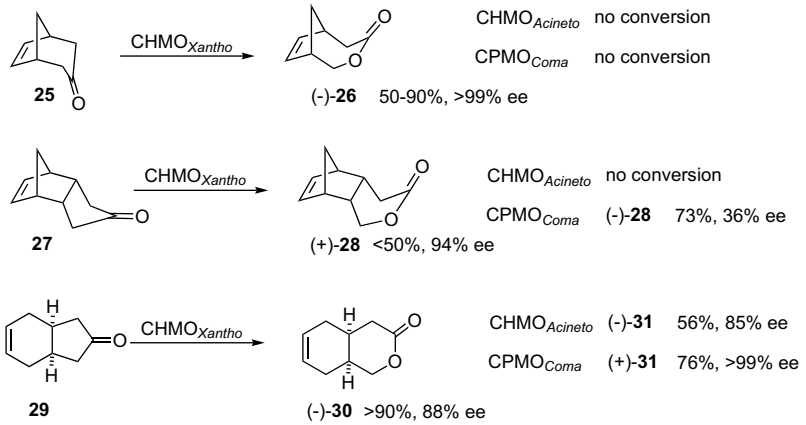


Figure 10. $CHMO_{Xantho}$ oxidizes sterically demanding bi- and tricyclic prochiral ketones to the corresponding lactones in good yields and generally with very high enantioselectivity. In some cases it is the only enzyme identified thus far that can accomplish a transformation (for example, the conversion **25** to **26**). In other cases, it provides an otherwise unavailable enantiomer (**27** to **28**) or a better yield (**29** to **30**).

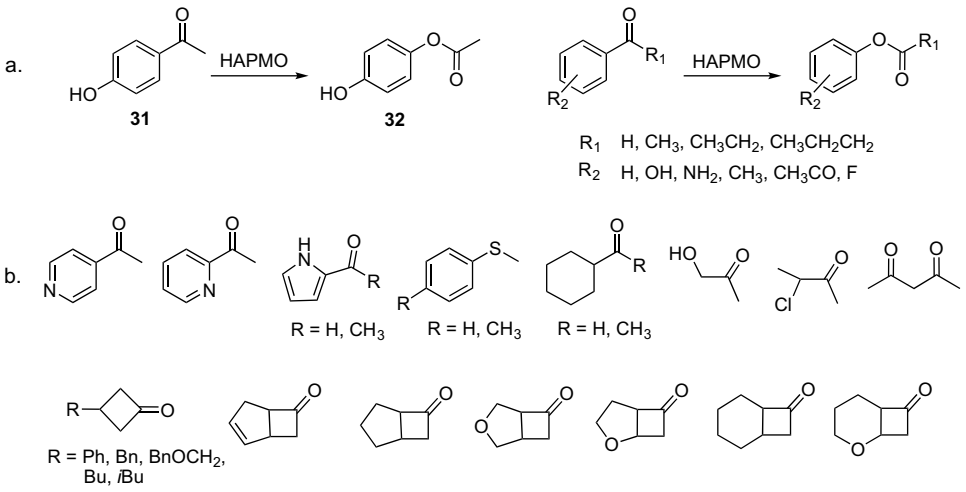


Figure 11. HAPMO converts a variety of substrates: acetophenones, heteroaromatic ketones, sulfides, and certain aliphatic ketones. The best results are observed with acetophenones and propiophenones. The heteroaromatic and other compounds are all converted by HAPMO in low to moderate yields. Oxidation of sulfides gives essentially enantiopure sulfoxides.[74b]

converted quantitatively to the corresponding lactones the conversion of longer chain ketones was low and hexadecane-2-one was not a substrate (Fig. 14). The ketones with carbon chain shorter than C8 were converted but the yields were low because of the loss of product through evaporation. The earlier reported BVMO from *Pseudomonas cepacia* that preferentially oxidizes C12 to C14 chains is the only other BVMO with similar substrate specificity.[101]

4.7.5. Phenylacetone monooxygenase (PAMO)

While the majority of synthetically useful enzymes were discovered by screening large numbers of different microbial strains against the desired substrate such as, for example, cyclohexanone or bicycle[3.2.0]hept-2-en-6-one, phenylacetone monooxygenase (PAMO) was discovered by design to find a more thermostable Baeyer-Villigerase.[102] To achieve that the genomes of multiple thermophilic bacteria were searched using a protein sequence motif common to known BVMOs (see Section 5.1). Two putative BVMO genes were identified in a moderately thermophilic soil bacterium *Thermobifida fusca* that grows at 55 °C. One of the two putative BVMOs showed high sequence identity with the two well established Baeyer-Villigerases, CHMO (40%) and SMO (57%), and was further tested. Expression in *E. coli* and subsequent characterization and screening showed it to accept aromatic, aliphatic ketones and sulfides with varying degrees of selectivity.[102] The enantiopreference of the enzyme, also substrate dependent, is generally (*S*) for esters and ranges from 98% ee for the (*S*)-configuration for benzyl ethyl sulfoxide to 80% ee for the (*R*)-configuration for methyl 2-phenylethyl sulfoxide.[102]

Preliminary studies have shown that PAMO is particularly successful in the oxidations of aromatic ketones and merits further study and development. PAMO and HAPMO, the two enzymes that readily accept aromatic ketones, were reunited in an exploration of kinetic resolution of racemic ketones. Both BVMOs were tested, using isolated enzymes in the Baeyer–Villiger oxidation of a prochiral ketone, 4-methylcyclohexanone, but the conversions and enantioselectivities were low. The attempts at kinetic resolution of racemic 2-methylcyclohexanone gave equally poor results. The substrate was oxidized by both enzymes, with PAMO being generally faster than HAPMO, but the observed (*S*) selectivity was consistently very low. The oxidations of aromatic aldehydes and especially aromatic ketones, on the other hand, were more successful. In the oxidation of 2-phenylpropionaldehyde (±)-**2a** catalyzed by PAMO and HAPMO under the same experimental conditions, moderate to low selectivities and enantiopurities were obtained (Table 2). The best results were obtained in the transformations of phenyl ketones where, under optimized conditions, a very high resolution could be achieved. (*S*)-Esters and (*R*)-ketones could be prepared via transformations with both enzymes but the reactions carried out with PAMO were faster and more selective than those performed with HAPMO (Table 2).[103]

4.7.6. Cyclopentadecanone monooxygenase (CPDMO)

Cyclopentadecanone monooxygenase (CPDMO), a new and synthetically promising addition to the family of BVMO reagents, was isolated from *Pseudomonas* strain grown on cyclopentadecanol and cyclopentadecanone. It was purified, characterized, and overexpressed in *E. coli*.[104] Substrate profiling indicates that CPDMO is a versatile enzyme capable of converting a variety of large (C15) and small (C6, C5) cyclic and bicyclic ketones, often with excellent enantioselectivity. Several representative examples are shown in Table 3. It is noteworthy that this enzyme converts 4-*t*-Bu-cyclohexanone with good yield and a very high enantioselectivity. It is possible that CPDMO may also accept other long and/or bulky

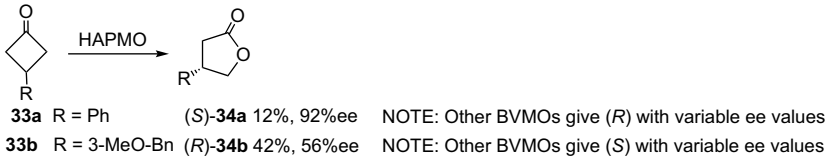


Figure 12. HAPMO-catalyzed oxidation of 3-phenyl-cyclobutanone **33a** gives lactone (*S*)-**34a** in low yield but with high enantiopurity. All other BVMOs give the (*R*) enantiomer with exception of $CPMO_{Coma}$ that gives (*S*) but with a very low enantiomeric excess. In the case of **33b** the situation is reversed HAPMO gives the (*R*) while all other BVMOs give (*S*)-**34b**.[35]

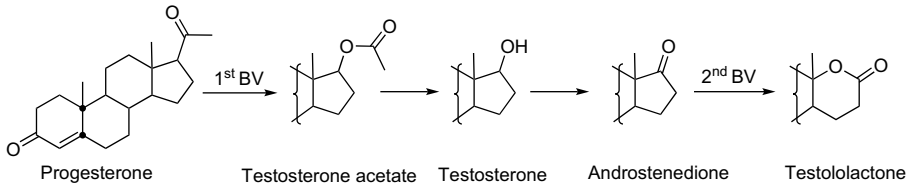


Figure 13. Metabolism of progesterone was established by isolation of the intermediates. Testosterone acetate intermediate was discovered when progesterone was incubated with *Cladosporium resinae*.[97] It was not observed in fermentation with *Penicillium lilacinum* because of high levels of esterase expressed in that organism.[96]

substituents in that position—an achievement that is not easily duplicated by other BVMOs.

With a growing collection of BVMOs, finding a suitable bioreagent for enantio- or regioselective Baeyer–Villiger or sulfide oxidation of a target substrate is becoming a reality. Evidently, further profiling is needed to establish the full potential of several new BVMOs.

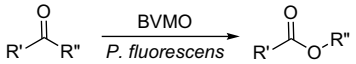
5. In search of new bioreagents

5.1. Genome mining

Excellent as they are, bioreagents discussed so far do not fulfill all the needs of synthetic chemistry. Despite many successes there are substrates that are not accepted by any of the currently available BVMO overexpression systems, or the regio- and enantio-selectivities of the conversions are unacceptably low. It is true that a number of Baeyer–Villiger monooxygenases of bacterial or fungal origin described in the literature have not yet been cloned and overexpressed, and even fewer enzymes have been screened for their substrate acceptance and selectivity. When explored, these enzymes will expand the repertoire of BVMOs and fulfill some, but not all, needs. This means that the search for the best enzymes for a particular transformation will continue. Originally, whole organisms, usually bacteria and fungi were screened to identify the biocatalyst that can catalyze the desired transformation, and although many excellent bioreagents (such as CHMO) have been derived from whole organism screening, this strategy is purely empirical and requires often difficult purification of low abundance proteins prior to gene cloning and overexpression.

Recent studies of flavin-containing monooxygenases[105] and the identification of the characteristic signature sequence FXGXXXHXXXW(P/D) of BVMOs[106] allowed for a new way, referred to as genome mining, of finding synthetically useful enzymes. The introduction of routine genome sequencing has dramatically expanded the inventory of putative protein sequences that can be searched for the residues, which are crucial for catalysis of Baeyer–Villiger reaction. For example, a search by the sequence motif of the genomes of thermophilic microorganisms resulted in the discovery of thermostable phenylacetone monooxygenase, PAMO, in actinomycete *T. fusca*.[102] In general, the genome searches performed by a pattern-hit (PHI) BLAST search of the database[13] showed that type I BVMO sequences are present in approximately 15% of all sequenced microbial genomes,[13] but not in Archaea, or plant and human genomes.[13] The combination of BVMO-identifying sequence motif with selection of only those protein sequences that share a significant sequence similarity with the known BVMOs prevents retrieval of flavin-dependent enzymes that are *not* BVMOs. A search performed in 2002 yielded 68 putative microbial BVMOs, confirming that a large pool of unexplored BVMOs is available for biocatalytic explorations.[13] Apart from rapid identification of many prospective bioreagents the advantage of genome mining using signature sequence is that it identifies the gene of a putative protein. The overexpression of that gene can be itself used in the screening process, and can be used to provide large quantities of isolated protein for research and screening.

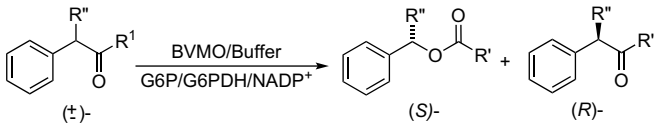
It is important to note that many of the putative BVMOs are found in genomes from pathogenic bacteria such as *Mycobacterium tuberculosis*, which contains six such sequences. One of these enzymes, responsible for activation of antitubercular drugs by



R'	R"	Time hrs.	Conversion %
CH_3	C_6H_{13}	8	38
CH_3	C_8H_{17}	19	100
CH_3	C_9H_{19}	20	92
CH_3	$C_{10}H_{21}$	19	83
CH_3	$C_{11}H_{23}$	20	68
C_2H_5	C_7H_{15}	19	100
C_3H_7	C_6H_{13}	19	100

Figure 14. The BVMO from *P. fluorescens* expressed in *E. coli* JM109 was used to catalyze oxidation of chain ketones shown above. The transformations were carried out at 30 °C for 19–20 h. In the case of octan-2-one exact conversion could not be determined beyond 8 h fermentation time because of the loss of the substrate and product due to evaporation.

Table 2
PAMO- and HAPMO-catalyzed oxidations of racemic phenyl aldehyde and phenyl ketones using isolated enzymes and NADPH recycling[103]



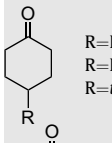
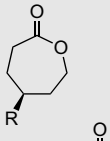
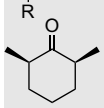
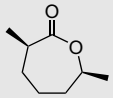
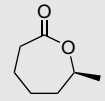
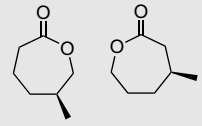
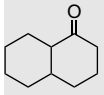
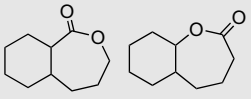
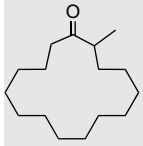
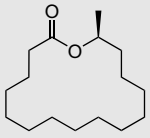
3t: R' = H, R" = Me
39: R' = Et, R" = Me
41: R' =Me, R" = Me
43: R' =Me, R" = Et
45: R' =Et, R" = Et

38: R' = H, R" = Me
40: R' = Et, R" = Me
42: R' =Me, R" = Me
44: R' =Me, R" = Et
46: R' =Et, R" = Et

Substrate (racemic)	BVMO	pH	*T* °C	Time (h)	Conv.[a] (%)	ee (%)[b] *R* ketone	ee (%)[b] *S* ester	*E*[c]
37	PAMO	8	20	8	36	50	88	25
37	HAPMO	8	20	30	9	9	88	17
39	PAMO	8	30	0.75	21	27	99	>200
39	HAPMO	8	15	14	50	98	97	>200
41	PAMO	8	20	1	27	36	98	188
41	HAPMO	8	20	14	30	43	97	137
43	PAMO	8	20	1	32	45	99	>200
43	HAPMO	8	20	14	45	78	96	117
45	PAMO	8	20	1	51	98	95	179
45	HAPMO	8	20	14	52	98	90	87

[a] Conversion, $c=ee_S/(ee_S+ee_P)$.
[b] Determined by GC.
[c] *E*-value, Ref. 77.

Table 3
Baeyer–Villiger oxidation catalyzed by CPDMO

Substrate	Conversion[a] 20 h (%)	Product lactone	Selectivity[b]
 R=Me R=Et R=*t*-Bu	54 74 68	 R=Me R=Et R=*t*-Bu	99% ee (*S*) 99% ee (*S*) 99% ee (*S*)
	74		99% ee
	46		99% ee (*S*)
	60		1 (96% ee):3 (96 % ee)
	80 (trans)		1 (99% ee):4 (33% ee)
	10		59% ee (*S*)

[a] Conversion is based on the EtOAc-extracted sample determined by GC or chiral-phase GC analysis and referred to an internal standard.
[b] Selectivity refers to regioisomeric lactone ratio, and the given enantiomeric excess values are based on determinations of beta-Dex 225 chiral-phase column GC analysis.

catalyzing sulfoxidation reaction,[107] was overexpressed in *E. coli* and tested in oxidations of fused bicyclic ketones.[108] It was found effective in resolving this type of racemic starting materials—with one enantiomer oxidized to single regioisomeric lactone while its enantiopure antipode remains unreacted (Fig. 15). The potential synthetic value of enzymes from highly pathogenic organisms emphasizes the importance of the overexpression systems in *E. coli* that are not only benign, but also produce large quantity of the desired enzyme in the cell, minimizing the possibility of unwanted side reactions.

5.2. Models

The success of biotransformations ultimately rests on the properties of available enzymes. These enzymes evolved to serve a specific metabolic function of their parent microorganism rather than the needs of a chemist. From the chemist's perspective it often means that once the best enzyme for a particular synthetic task has been identified it may be necessary to make it 'better.' In many cases, a route to a better bioreagent requires a design, or rather re-design, of the best-available-for-the-task enzyme. If one were to re-design an enzyme for a particular substrate or a particular task, such as enhancement of enantioselectivity, one has to know something about its catalytic mechanism and its structure. When this information is not available the only way to modify an enzyme is Nature's way—that is through evolution. Enzymes' exquisite adjustment to their physiological roles has taken millions of years; it is too long for a chemist to wait; she needs a shortcut. As usual, Nature provided a model: formation of a large number of variants through mutation and subsequent selection of the 'fittest for the task' mutant. Such evolution can be carried out in the test tube with enhanced speed thanks to modern molecular biology methods. Termed 'the directed evolution', it provides a powerful technique for the development of new or improved biocatalysts without knowledge of enzyme's structure or catalytic mechanism.[109]

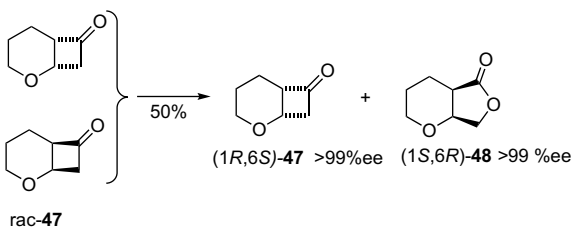


Figure 15. Highly efficient resolution of racemic compound **47** is accomplished using *E. coli* expression system for monooxygenase from pathogenic bacterium *M. tuberculosis*.[108]

No three-dimensional structure of any of the BVMOs was available until the publication of the X-ray structure of phenylacetone monooxygenase PAMO in 2004.[110] Before that, in the absence of three-dimensional structure many attempts were made toward development of models that could explain exceptional substrate acceptance and selectivity of CHMO-catalyzed conversions and could be used to predict transformations of the new and untested substrates. Nearly all models focused on $CHMO_{Acineto}$, the best characterized member of the BVMOs family, since its catalytic mechanism had been elucidated and its large substrate base provided information on the size as well as polar and spatial requirements of the active site.

CHMO mechanistic studies[15a,16,42] showed that biological Baeyer–Villiger reaction possesses the same characteristics and requirements as its chemical counterpart including the formation of the Criegee intermediate. The fact that flavin, which as **4a**-hydroperoxyflavin peroxide assumes the role of a peracid (see Fig. 1), remains tightly bound within the active site throughout the entire catalytic cycle had important implications for the development of models because it localized the position of the intermediate within the CHMO's active site. Product analysis in conjugation with potential flavin binding produced the first active site models [111,112] that allowed for the prediction of the absolute configuration of a newly created stereogenic centers from the CHMO-catalyzed oxidation of fused bicyclic and prochiral ketones. Furstoss' model proposed a cube in which flavin occupied a fixed position and where the stereoselectivity was determined by the ability of the active site to distinguish groups of different sizes.[112]

In Taschner's model the peroxy ion, formed on the *re*-face of the flavin molecule, attacks the carbon atom of the substrate carbonyl group leading to the formation of a Criegee intermediate in which the C–O–O–flavin bond is in the equatorial position. The proposed existence of a single binding site for the two enantiomers of a substrate was predicated on the facts that: (a) the amino acid sequence of CHMO[17] indicated only single binding sites for each of the cofactors (FAD and NADPH), and (b) both enantiomers of an inhibitor bicyclic thiolactone caused identical inhibition.[113]

Later, Kelly and co-workers[114] proposed a model based on the 'central dogma' of the mechanism for the Baeyer–Villiger reaction that states that the migrating bond in the Criegee intermediate must be aligned antiperiplanar to the oxygen–oxygen bond of the peroxide. This model elegantly explained regiodivergence of enantiomeric fused bicyclic ketones (**10**-*S*,*R* and **10**-*R*,*S*) and at the same time demonstrated that both antipodes can fit into an active site of the enzyme by predictably oxidizing a tricyclic ketone **50** that structurally corresponds to the superposition of the two enantiomers of ketone **10** (Fig. 16). In this model the positions of the three oxygen atoms participating in the rearrangement, fixed in space relative to each other, define an ensemble capable of

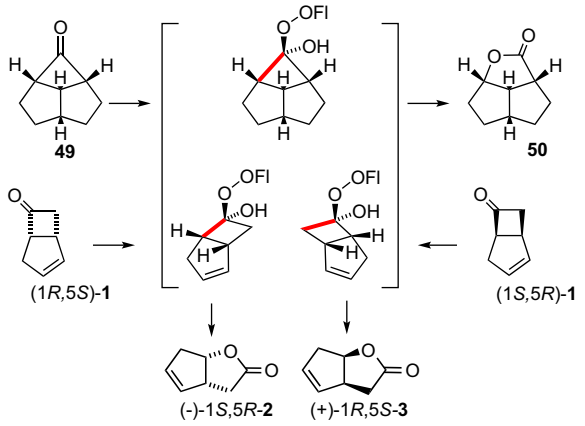


Figure 16. Kelly's model rationalized regiodivergence of the CHMO and other BVMOs-catalyzed oxidations of racemic ketone **1**. The conversion of ketone **49** (a structural superposition of the two enantiomers of ketone **1**) to lactone **50** not only provided support for the model but also confirmed earlier assumptions for the existence of a single active site in the BVMO enzymes. Criegee intermediates involved in both BV oxidations are shown in a bracket with the migrating bonds outlined in red.

undergoing *an enantioselective* Baeyer–Villiger reaction. The potential intermediates are non-superimposable even if the substituents (R_1, R_2) are identical, and a Baeyer–Villiger reaction that occurs under stereoelectronic control must always proceed via a chiral transition state even with prochiral ketones.

To create a model that could explain and predict the stereoselectivity of the oxidation of organic sulfides to optically active sulfoxides catalyzed by CHMO, Ottolina and co-workers applied molecular modeling software to minimize the total energy of over 30 different sulfides. The minimized structures were superimposed along the C–O peroxide bond to define various binding pockets that emerge from inserting the minimized structures inside a 'box' comprised of several cubes.[115] The stereopreference of CHMO-catalyzed oxidation of benzylmethyl sulfides substituted in *p*-position with groups of different polarity extended the usefulness of the model by locating inside the enzyme active site model electron-rich and electron-poor regions.[116] When applied to CHMO-catalyzed Baeyer–Villiger reactions Ottolina's model gave results that were consistent with and expanded the analyses of the earlier models.[117] In sum, this excellent model is very precise but requires computer and molecular graphics.[12]

To rapidly evaluate the outcome of a proposed biotransformation, Prelog's[118] diamond lattice model was adapted to CHMO-catalyzed oxidations.[27] Based on the stereoelectronic demands of Baeyer–Villiger reaction and the assumption that flavin occupies a fixed position within the active site, the known cyclohexanone substrates were overlaid onto the diamond model in which the C–O–O–flavin bond is fixed in equatorial position as shown in Figure 17. This simple model that can be applied only to substrates containing sp^3 centers only nonetheless has proven to be useful for predicting the selectivity of CHMO vis-a-vis new substrates.

In the absence of the X-ray crystal structure of CHMO and any other BVMO, the above models have been very helpful in visualizing of the CHMO-catalyzed biotransformations and in predicting the selectivity in oxidation of specific substrates.

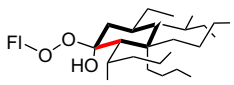


Figure 17. Diamond lattice model of the allowed sizes and substitution pattern. The carbon–carbon bond, which is shown red migrates during fragmentation of the intermediate.

A recent publication of the three-dimensional structure of phenylacetone monooxygenase PAMO from *T. fusca*[110] opened the door to a better understanding not only of the PAMO itself, but of the whole family of BVMO enzymes, and allowed for a 'rational' design or re-design of bioreagents.

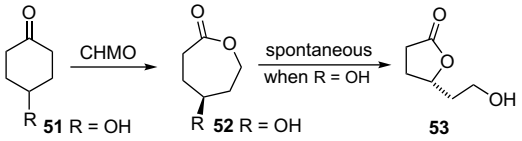
5.3. Directed evolution of CHMO

The directed evolution of an enzyme can lead to improvement in substrate specificity, thermal stability, tolerance to organic solvents, and most recently enantioselectivity, without the knowledge of that protein's structure.[109] Technically speaking, directed evolution involves random mutagenesis of a target gene and overexpression in a suitable host such as *E. coli* followed by screening for the desired characteristics. Repeated cycles exert evolutionary pressure: the most promising mutants identified via screening are used as starting points for further rounds of genetic mutation, expression, and screening until a mutant possessing all of the desired characteristics is identified.[109,119] Among several mutagenesis methods available,[120,121] error-prone polymerase chain reaction epPCR[122] is practical and frequently used in directed evolution. Screening for enzyme properties such as activity, thermal or solvent stability is reasonably simple; screening for enantioselective enzymes produced in directed evolution, however, is more complicated. The number of variants produced in each evolutionary cycle is very large while beneficial mutations are few. Screening of thousands of mutants for enhanced enantioselectivity requires development of efficient and rapid methods of their evaluation as catalysts in asymmetric reactions. Since none of the screening methods is universal, the development and adaptation of a fast assay (a high-throughput screening methodology) is a major challenge in each evolutionary project.[121] Initially, directed evolution of enantioselectivity was limited to lipases[123] and epoxide hydrolases[124] and a number of effective assays based on UV/vis and other spectroscopic methods was developed for screening enantioselectivity of mutant-catalyzed transformations.[121a]

The first directed evolution of a BVMO enzyme was performed in an attempt to improve enantioselectivity of $CHMO_{Acineto}$.[125] Highly enantioselective, and predictably (*S*) selective in the oxidation of several 4-substituted cyclohexanones, CHMO converted 4-hydroxycyclohexanone **51** to lactone *R*-**53** with only 9.6% enantiomeric excess (Table 4).

Since at the time no X-ray data or homology models were available, the only way to improve CHMO oxidation of ketone **51** (R=OH) was via directed evolution. In this case several libraries of mutants were produced using epPCR. The mutant genes were inserted into the *E. coli* host[27] and plated on agar. A colony picker was used to harvest cells and place them in the 96 deep wells of

Table 4
CHMO-catalyzed Baeyer–Villiger oxidations of 4-substituted cyclohexanones[12]



R	Method	Lactonic product yield; configuration; ee
Methyl	CHMO/baker's yeast	83%; (*S*); >98% ee
Ethyl	CHMO/baker's yeast	74%; (*S*); >98% ee
Propyl	CHMO/baker's yeast	63%; (*S*); 92% ee
Allyl	CHMO/baker's yeast	62%; (*S*); 95% ee
Isopropyl	CHMO/baker's yeast	60%; (*S*); >98% ee
Methoxy	*Acinetobacter*	76%; (*S*); >75% ee
Hydroxy	Isolated CHMO	73%; (*R*); 9.6% ee

a microtiter plate containing growth medium. The growing cells were used in the transformation of 4-hydroxycyclohexanone. The product was extracted with ethyl acetate and the extracts were analyzed by chiral GC set-up, which allows approximately 800 ee determination per day. The whole process was carried out using robotics. Among 10,000 mutants screened there were a few dozens hits: mutants with enhanced *R* enantioselectivity and mutants with reversed, high *S*, selectivity. Of the eight mutants sequenced, variant 1-K2-F5 producing lactone (*S*)-**53** (79% ee) had a single amino acid exchange: phenylalanine in position 432 was replaced with serine (F432S). In the second round of evolution mutant 1-F1-F5 (L143F) showing a modest enhancement of *R*-selectivity (*R*)-**53**, 40% ee, became a starting point. Screening 1600 mutants led to a highly *R*-selective mutant 2-D19-E6 with three amino acid exchanges (E292G, L435Q, T464A) in addition to its original L143F mutation. 2-D19-E6 mutant can convert **51** to (*R*)-**53** with greater eantioselectivity (90%) but its stability is low.

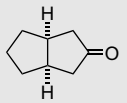
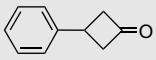
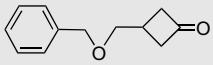
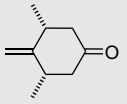
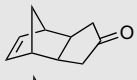
The *S*-selective 1-K2-F5 mutant was tested in Baeyer–Villiger oxidations of several 4-substituted cyclohexanones (Me, Et, Cl, Br, I, OCH_3) and was shown to convert these substrates with the same or enhanced *S* enantioselectivities of 95–99% ee[125] as the wild type CHMO.[32b,81] Evaluation of several best mutants as catalysts in Baeyer–Villiger oxidations for a series of 4-substituted and 4,4-disubstituted cyclohexanones further confirm superiority of the 1-K2-F5 as an enantioselective, robust, and versatile reagent for Baeyer–Villiger oxidations.[126] The subsequent screening of this mutant against a range of other cyclic and bicyclic ketones showed it to be as active and selective as the wild type CHMO and in some cases better.[127] Several of the results are shown in Table 5. It is interesting to note that tricyclic ketone, entry 5, not accepted by the wild type CHMO was oxidized by mutant 1-K2-F5 with high enantioselectivity.

Since CHMO catalyzes oxidation of sulfides to sulfoxides, the original library of 10,000 clones (explored in the BV reactions) was screened to identify potential catalysts for oxidation of thioether **54**, which is a poor substrate for wild type (WT) CHMO.[128] Screening was carried out using chiral HPLC adapted to processing 800 ee determinations per day,[121a] and several variants with high *R* and *S* ee values were discovered. Most of the mutants were different from those identified in the BV screening. However, one of the best performing variants carried a single F432S mutation identical to that found in mutant 1-K1-F5 earlier shown to be a highly enantioselective biocatalyst for Baeyer–Villiger oxidation of a wide range of ketones.[125–127] Three representative mutants are listed in Table 6. It is amazing that the same mutant was identified twice in the course of screening the same library with two different substrates corresponding to two different reactions.

Although several of the identified mutants gave high ee values, the overoxidation to sulfone was observed in most cases. Careful analysis of the products ratios in the course of the reaction catalyzed by mutant 1-D10-F6 showed that an increase in the formation of sulfone **56** was accompanied by the enrichment of (*R*)-**55** sulfoxide. This indicates that the preferential formation of (*R*)-**55** is aided by preferential oxidation of the (*S*)-**55** sulfoxide to sulfone. An analogous effect was observed in the case of other mutants. One of the *S*-selective mutants with significant (26%) sulfoxide production was used as a starting point in a second round of epPCR in an attempt to eliminate sulfone formation. A library of 1600 clones was screened for high *S*-selectivity and low sulfone-formation, leading to the discovery of a new variant with selectivity of 99.8% ee in favor of (*S*)-**55** and negligible (<5%) quantity of **56**.

The above experiments demonstrated the power of directed evolution in the development of new bioreagents that carry out desired reactions with excellent selectivity. The discovery that the evolved mutants showing highest enantioselectivity for the specific reaction can also be better catalysts for other substrates is an additional bonus. Another important benefit of the directed evolution, particularly in the absence of the enzyme's X-ray crystal structure is that it allowed to identify some of the amino acids' residues intimately involved in the catalytic process, the 'hot spots.' Thus, in oxidation of 4-hydroxycyclohexanone, several mutants carried the mutation at the 432 and 143 positions. The most successful and versatile mutant Phe432Ser was also highly enantioselective in the oxidation of methyl-*p*-methylbenzyl thioether to (*R*) sulfoxide (98.7%). These results showed that enhanced selectivity for one substrate was not at the expense of the enzyme's robustness and broad substrate tolerance. Furthermore, the directed evolution of the CHMO enzyme with an unknown active site was successful in identifying one residue crucial for enantioselectivity control, thus placing it within the active site region.[126]

Table 5
Profiling of mutant 1-K2-F5 (Phe432Ser)

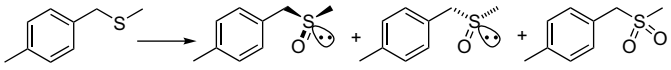
Entry	Substrate	Bioreagent	+/−[a]	ee (%)	Conversion (%)
1		Wild type	−	89	>90
		1-K2-F5	−	94	>90
2		Wild type	−	62	>90
		1-K2-F5	−	96	>90
3		Wild type	+	53	>90
		1-K2-F5	−	83	>90
4		Wild type	+	96	>90
		1-K2-F5	+	99	>90
5		Wild type	n.a.	n.a.	n.c
		1-K2-F5	−	90	50
6		Wild type	−/−	44:>99[b] (70:30)[c]	>90 >90
		1-K2-F5	−/−	65:>99[b] (58:42)[c]	

n.a. not applicable; n.c. no convertion.
[a] Sign of specific rotation.
[b] ee of 'normal lactone' to ee of 'abnormal lactone'.
[c] Ratio of 'normal lactone' to 'abnormal lactone'.

5.4. 'Rational design' of new or better bioreagents

A milestone in the development of Baeyer–Villiger monooxygenases was the crystallization and X-ray analysis of phenylacetone monooxygenase (PAMO) by Mattevi and co-workers.[110] None other of many purified BVMOs produced crystals suitable for

Table 6
High performing mutants from the directed evolution of $CHMO_{Acineto}$[128]



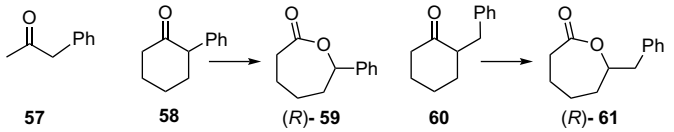
Mutant	Amino acid exchanges	Yield (%) of sulfoxide **55**	Configuration	ee (%)	Sulfone **56** (%)
WT CHMO	—	75	(*R*)	14.0	<1
1-D10-F6	Asp384His	75	(*R*)	98.9	7.9
1-K15-C1	Phe432Ser	55	(*R*)	98.7	20.0
1-C5-H3	Lys229Ile, Leu248Pro	77	(*S*)	98.1	5.6

X-ray analysis. PAMO was crystallized with a bound FAD cofactor, and although it was not possible to obtain crystal of the enzyme with both FAD and NADPH in place, the NADP binding site could be deduced from the comparison with the NADPH complexes of flavoenzymes with similar folding topology.[110] The existence of the three-dimensional structure of a Baeyer-Villigerase allowed the construction of homology models of CHMO and other BVMOs and opened the door to interpretation of earlier results and new 'rational' designs of BVMO biocatalysts.

PAMO, the only BVMO with a known three-dimensional structure, has been an attractive target for rational redesign. PAMO possesses many characteristics highly valued in enzyme: it is thermostable, tolerates organic solvents and is highly active. On the other hand, its substrate base is limited to linear aromatic sulfides and ketones and its enantioselectivity is frequently low. It is not surprising, therefore, that the principal efforts in rational design of BVMOs have been focused on this enzyme. What if one could redesign PAMO to be more like CHMO with its exceptional acceptance range, its high enantioselectivity, while retaining its own superior robustness? The first attempt at rationally improving PAMO was modeled on CHMO.[129] Comparison of the crystal structure of PAMO with the homology model[130] of CHMO showed that the most apparent difference between the two enzymes is a three amino acid (Ser-441, Ala-442, Leu-443) bulge in the loop near the active site of PAMO that is absent in CHMO. The three enzyme variants generated by removing one or two of these amino acids retained thermostability of WT PAMO and showed increased acceptance of compounds **58** and **60**, but catalytic efficiency of the conversion of the parent substrate phenylacetone **57** was diminished. Of several compounds screened, only ketones with the phenyl ring near the carbonyl group, **58** and **60**, were accepted by the best of three mutants in which Ser-441 and Ala-442 were deleted. Thus, the partial deletion of the 'bulge' turned PAMO into a highly effective phenylcyclohexanone monooxygenase without significantly increasing or diversifying its substrate base (Table 7).

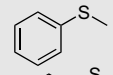
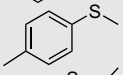
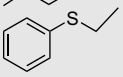
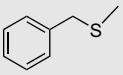
Another attempt at altering substrate specificity of PAMO used CPMO as a model.[131] The two enzymes share 41% sequence identity but display very different substrate acceptance specificities. Comparison of the amino acid residues in PAMO's structure with the homology model of CPMO showed that only three residues were fundamentally different: Gln152, Leu153, and Met446 in PAMO corresponded to Phe156, Gly157, and Gly453 in CPMO. Of the single, double, and triple mutants prepared the most useful turned out to be able to carry an exchange of a more bulky, non-polar methionine for a non-polar, sterically undemanding glycine (Met446Gly). The mutant screened against several aromatic ketones, aromatic sulfides, and aromatic amines showed the most promise in oxidation of sulfides, Table 8. The best results were obtained when the phenyl group is substituted on the sulfur atom (entries 1–3). Mutant's selectivity of benzylmethyl sulfide, where the phenyl is separated form sulfur by the methylene group is rather low; this is convenient, however, since its selectivity is *R* therefore opposite of the high *S* attained with PAMO. Since benzyl sulfide with short alkyl chains is an excellent substrate for PAMO (entry 4), the ability of mutant Met446Gly to convert phenyl sulfides with high enantioselectivity, complements nicely the repertoire of PAMO.

Table 7
Kinetic resolution of substrates **58** and **60** by a mutant with deletion of Ser-441 and Ala-442[129]



Substrate	Enzyme/variant	*E*	% Conversion 24 h	Preferred lactone
58	WT PAMO	1.2	10	*S*
58	Mutant	100	91	*R*
60	WT PAMO	—	0	—
60	Mutant	>200	40	*R*

Table 8
Comparison of sulfoxidation catalyzed by WT PAMO and mutant Met446Gly

Entry	Substrate	WT PAMO		M446G	
		Conversion (%)	ee (%)	Conversion (%)	ee (%)
1		94	41 (*R*)	>99	93 (*R*)
2		69	6 (*R*)	>99	92 (*R*)
3		94	6 (*S*)	>99	95 (*R*)
4		>99	98 (*S*)	>99	59 (*R*)

Directed evolution requires access to expensive equipment and is often very labor-intensive. It does, however, permit to identify mutants with specific characteristics; in other words: 'you get what you screen for.' The rational design could relieve the need for screening large numbers of mutants but it requires an in-depth knowledge of the structure–function relationships and consideration of factors such as entropy to predict the effects of point mutation on enzyme's performance.[132] As practiced, rational design is based on notions derived from experience. For example, changes in enantioselectivity, substrate specificity, and catalytic activity are most likely to be beneficial when amino acids located in or near the active site, 'first shell residues,' are modified, while other properties (stability and activity) can be improved by mutations either near or far from the active site.[133] Also, the most successful mutations are inspired by comparison with related enzymes possessing the desired attributes.[129,131] The results obtained using the 'rational' approach can be very interesting and useful, as was shown in the two examples of rational design of PAMO, but they are often not what was expected.

Then, there is the question of deciding, which enzyme is the best candidate for modification. That, of course, depends on the purpose of evolving or re-designing an enzyme. Certain enzymes transform a single substrate or a narrow range of structurally related compounds, others, 'the promiscuous' enzymes, are capable of accepting and converting a variety of substrates. The same holds for reactions catalyzed by enzymes; many catalyze not just their 'natural' reaction but an alternative reaction as well (for example, Baeyer–Villiger oxidation and sulfoxidation). The majority of promiscuous enzymes identified thus far are thought to make use of the same active site configuration to catalyze the primary and other transformations, while small structural fluctuations within the active site allow it to accept multiple substrates or catalyze multiple reactions.[134] Both types of enzymes are represented among Baeyer-Villigerases: those with a relatively narrow specificity, like PAMO, or promiscuous ones like CHMO. It would seem that the first type may have a relatively rigid active site, while a promiscuous enzyme has a built-in 'structural fluctuation' (such as CHMO or CPMO) that may tolerate better mutations in the vicinity of the active site. It is possible that the promiscuous enzymes exist so that Nature (and chemists) can use them as an evolutionary starting point to obtain enzymes with new functions.

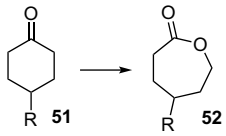
Semi-rational approaches to enzyme design combine the benefits of directed evolution and rational design.[135,136] In this

methodology, the 'hot spot residues' identified through directed evolution are subjected to saturation mutagenesis (where all 20 natural amino acids are tested at the target residue), giving rise to 'smart or focused libraries'.[136] An excellent example of the effectiveness of the semi-rational approach is illustrated by the modification of enantioselectivity in *Pseudomonas aeruginosa* lipase from E=1.1 in the wild type to E=51.[123] Smart, or focused, libraries have been created to improve the catalytic performance of enzymes, including enhanced enantioselectivity.[137] A particularly effective method in the generation of focused libraries is the Complete Active Site Saturation Test (CAST).[137,138] Based on the three-dimensional structure of an enzyme (X-ray or homology model), two or three amino acids whose adjacent side chains are localized within the binding pocket are randomized giving rise to libraries of mutants.[137] In the case of the initial library-generating mutants, which require further optimization, iterative CASTing,[138] or a combination of improved amino acid exchanges from two different mutants can be beneficial.[139] These tactics applied to lipases led to significant substrate acceptance[137,139] and to enhanced enantioselectivity in epoxide hydrolases.[138]

The 'mini evolution,' a cross between directed evolution and rational design, was conceived to generate mutants while reducing the costs and labor associated with directed evolution.[140] This semi-rational approach can be used when information about the residues in the close proximity of an active site is available. The combination of the available X-ray structure of PAMO and sequence alignment of CHMO and CPMO with PAMO that shows high homology, 39% and 40%, respectively, indicate that the structure of the three monooxygenases should be highly conserved. Indeed, the structural models[141] show the cores of the three proteins to be almost identical (Fig. 18).

Mini evolution applied to CPMO used modified CAST method to produce mutants. The two mutation sites in CPMO were based on the corresponding 'hot spots' identified in the CHMO evolution (see text above). The amino acid pairs Phe156/Gly157 and Gly449/Phe450 were used to create libraries A and B, respectively. Screening 150 clones from each library (about 65% coverage of the protein sequence space defined by the CAST) permitted identification of several mutants with much improved enantioselectivity, vis-a-vis a series of 4-substituted cyclohexanones. A few examples are shown in Table 9.[140,142]

Table 9
Baeyer–Villiger oxidation of 4-substituted cyclohexanones catalyzed by WT CPMO and selected mutants



Substrate	Bioreagent	Position	Mutation	Conversion (%)	ee (%)	Configuration
R=Me	WT CPMO	—	—	100	46	*R*
	A1A10	435/436	GlyPhe/GluIle	74	92	*R*
	B1A10	156/157	PheGly/Leu/Phe	89	91	*R*
R=Et	WT CPMO	—	—	100	36	*S*
	B1A10	156/157	PheGly/Leu/Phe	84	90	*R*
R=Pr	WT CPMO	—	—	100	36	*S*
	B1A10	156/157	PheGly/Leu/Phe	94	89	*R*
R=EtO	WT CPMO	—	—	100	37	*R*
	B1A10	156/157	PheGly/Leu/Phe	77	95	*R*
R=AcO	WT CPMO	—	—	81	5	*S*
	B1A10	156/157	PheGly/His/Leu	55	74	*R*

The directed and mini evolutions aimed toward improvement of enantioselectivity of CHMO and CPMO, combined with extensive substrate profiling, not only identified several high performance mutants but also provided insight into the origin of enantioselectivity toward 4-substituted cyclohexanones of the active sites of these enzymes. Study of CHMO, CPMO, and their mutants showed that the most beneficial mutations are at the 432 position in CHMO and at the 156/157 position in CPMO and that while mutations at 432 tended to improve the *S*-selectivity in CHMO,[126] mutations at 156/157 improved (*R*)-selectivity in CPMO, Table 9.[142] In the three-dimensional homology models [130] of CHMO and CPMO, these residues are located on the opposite sides of the catalytically essential[110,143] Arg327/344 (CHMO and CPMO, respectively) and in the proximity of the *re*-face of FAD as illustrated in Figure 19.

During enzymatic Baeyer–Villiger oxidation, the substrate interacts with FAD peroxide to give the Criegee intermediate.[42] At that time, the arginine moves out of the position that stabilizes (and blocks) the peroxy group and shifts into a position that allows it to stabilize the negative charge on the O^- of the intermediate.[110,129] The most effective stabilizations of the 4-*R* substituent on the

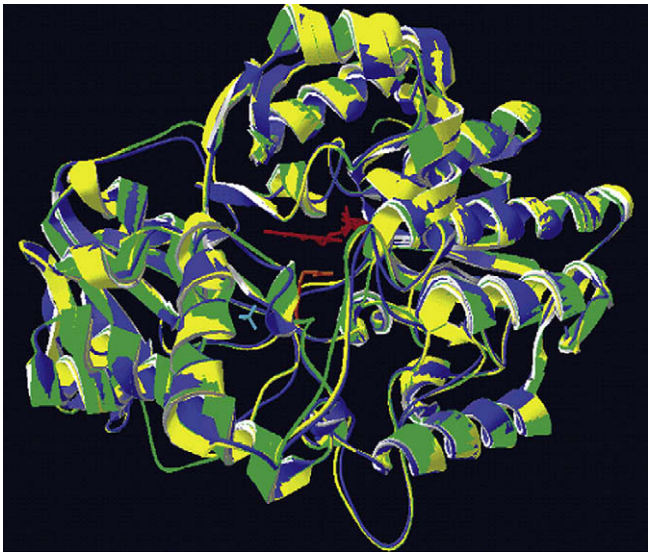


Figure 18. Homology models for CHMO (blue) and CPMO (green), shown in ribbon form, are almost identical with PAMO's X-ray crystal structure (yellow). The bound FAD is shown in red. The position of the phosphate of NADPH is simulated by a sulfate ion (turquoise), and the catalytically essential Arg337 residue is shown in orange.

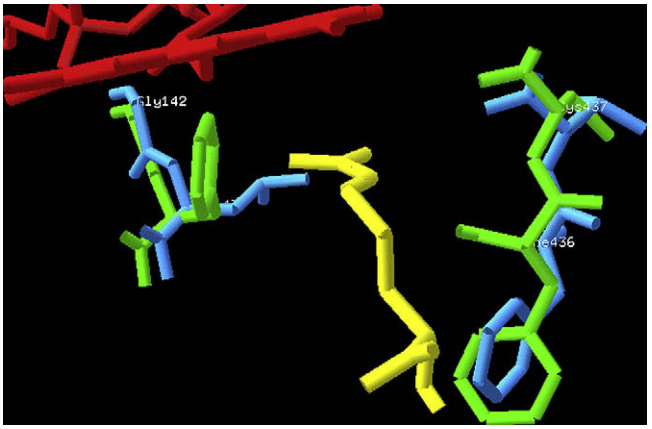


Figure 19. Relative positions of the key amino acid residues in the active site: FAD is shown in red; the catalytically essential Arg327/344 (CHMO/CPMO) is yellow. CHMO's residues Gly142 and Leu143 (blue) overlap with CPMO's Gly156 and Phe157 (green) defining one putative pocket. CHMO's Phe432 and Thr433 (blue) corresponding to CPMO's Gly449 and Phe450 (green) define the other pocket.

cyclohexanone in the 157 pocket leads to product lactone with configuration (*R*), while 4-*R* stabilization in 432 pocket gives the product lactone (*S*), as illustrated in Figure 20.[142]

Developments in the field of biocatalysis are clearly not limited to Baeyer–Villiger monooxygenases. The search for new and better oxidizing, reducing, and other potentially valuable bioreagents goes on. For example, in the family of reductases 'mining' of yeast genome led to identification of a large number of synthetically valuable reductases,[144–146] while a carbonyl reductase from *Sporobolomyces salmonicolor* (SSCR) with an unusual substrate range was shown to be a useful biocatalyst for the reduction of sterically bulky ketones.[147]

6. Development and optimization of experimental protocols

From the perspective of an organic chemist Baeyer–Villiger monooxygenases overexpressed in *E. coli* are user and environmentally friendly bioreagents for synthesis of enantiopure lactones, esters, and sulfoxides. The substrate base of the presently available BVMOs is quite vast, covering cyclic, alicyclic, aromatic ketones, and a broad range of sulfides. The protocols for testing suitability of bioreagents for the desired substrate oxidations are easy and require only standard equipment available in any chemistry laboratory. Maintenance and propagation of strains are equally simple and the only prerequisite is the access to −80 °C refrigerator. Maintained at that temperature most strains are active for months.

The real and perceived difficulties outlined in Section 1 have discouraged more general application of bioreagents; in addition, the transition from small-scale exploratory experiments to preparative or large-scale production has been considered as problematic. A number of groups aware of this, addressed the issue of making biocatalytic Baeyer–Villiger reactions not only accessible but also practical for chemists. Here, we outline how the experimental, whole-cell transformation procedures evolved to permit large-scale production of the desired products.

6.1. Whole-cell transformations

6.1.1. Transformations with growing versus non-growing cells

In the whole-cell-catalyzed Baeyer–Villiger oxidations performed under growing conditions bioconversion takes place during the logarithmic phase of the cells' growth, and the reduced form of the NADPH cofactor is regenerated during cell growth in the medium containing glucose. The growing-cell protocols are very simple and are suitable for screening and small-scale reactions. However, under growing conditions elevated concentrations of substrate and/or product can inhibit cell propagation. To minimize that problem, the concentration of substrate must be kept adequately low, thus limiting productivity of the transformation. To efficiently scale-up the Baeyer–Villiger reactions, Walton and Stewart developed a methodology for whole-cell *E. coli*/CHMO-catalyzed transformations to be performed under non-growing conditions.[148,149] The non-growing or resting cells have been used successfully before, generally in non-redox biocatalysis.[150] Adapting non-growing cells to bioconversions with NADPH-dependent enzymes requires conditions, which allow cellular metabolism to continue providing NADPH in the absence of cell division. The non-growing cells protocol of Walton and Stewart is simple. *E. coli*/CHMO cells are grown under typical growing conditions in the rich medium until they attain stationary phase, or the point at which dwindling nutrient supplies reduce bacterial growth and bacterial numbers stabilize. The cells are then harvested and re-suspended in a minimal salts medium lacking a nitrogen source. Nitrogen starvation inhibits cell replication but still allows the cell's biological machinery to regenerate NADPH (under glucose fed conditions) and, therefore, to act as a catalyst. The model oxidation of cyclohexanone to caprolactone performed with non-growing cells afforded 9.1 g/L product using a biocatalyst/substrate ratio of 0.63 g/g. Thus, the use of a non-growing cell strategy increased volumetric productivity approximately 20-fold with respect to the reactions carried out with growing cells.[148] The subsequent study established that the intracellular stability of CHMO and the rate of substrate transport across cell membrane, and not the supply of NADPH, controlled the reaction rate and duration.[149] Reduction of ethyl acetoacetate by non-growing *E. coli* cells overexpressing a yeast reductase (Gre2p) was even more efficient, providing 15.8 g/L from a biocatalyst/substrate ratio of 0.37 g/g.[149]

Baeyer–Villiger oxidations of 4-substituted cyclohexanones by *E. coli* expression systems for CHMO, CPMO and their mutants carried out under non-growing conditions, gave better conversions in shorter time than the parallel transformations with growing cells.[151] The most interesting observation was that several substrates, which did not react under growing conditions were converted to lactones under non-growing condition. In particular, CHMO mutant, Phe432Ser-catalyzed oxidations of 4-ethyloxy, 4-allyloxy, and 4-acetoxycyclohexanones gave essentially enantiopure (>99% ee) (*S*)-lactones with excellent conversions within 24 h. Under the same reaction conditions CPMO mutant Phe156Gly/Leu157Phe produced the (*R*) antipodes in good to excellent yields (Table 10). It appears, therefore, that the frequently encountered problem of substrate acceptance and/or conversion may, in many cases, be linked to the sluggish transport of substrate into the cytosol where the reaction takes place. The apparently more effective transport in the non-growing cells is likely related to the increased membrane permeability, which is caused by a high concentration of hydrophobic substrate and product in the fermentation mixture. These results suggest that bioconversions under non-growing conditions facilitate transport of substrate and product across membranes, and increase rates of the transformations. Since non-growing conditions are preferred for large-scale transformations in the reactor,[148] excellent conversions of the mutants indicate that these 'new designer bioreagents' can be used to prepare large quantities of lactonic products in scaled-up fermentation under non-growing conditions.[151]

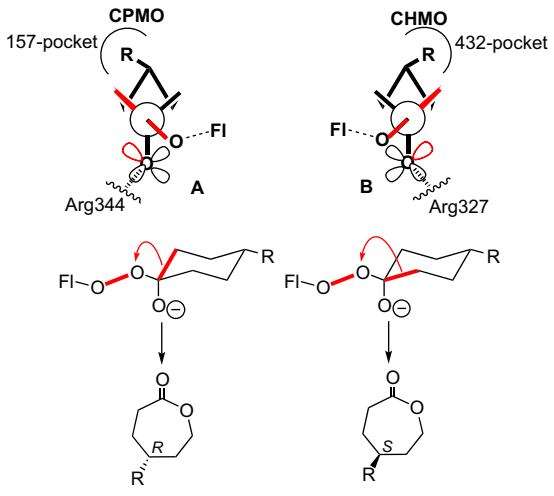
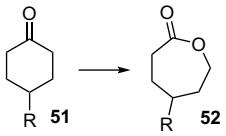


Figure 20. The diamond lattice model predicts that mutations of CPMO residues 156/157 (**A**) should improve the interactions with the 4-*R* substituent, therefore favoring the migration of the C–C bond (shown in red) leading to the (*R*)-lactone. The opposite is true when mutations occurred at the 432 position (**B**) in CHMO.[142]

6.1.2. Scale-up strategies

To provide a more productive and efficient process for whole-cell microbial Baeyer–Villiger oxidations that would allow

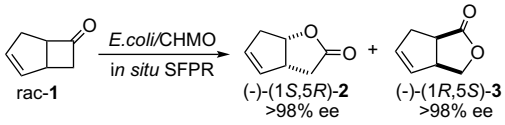
Table 10
Conversion of 4-substituted cyclohexanones with *E. coli*/CHMO and *E. coli*/Phe432Ser under growing and non-growing conditions[151]



Substrate growing conditions			Non-growing conditions		
R	WT-CHMO	Phe432Ser	WT-CHMO Conversion %[a] (ee%)[b]	Phe432Ser Conversion % (ee%)	CPMO: Phe156Gly/Leu157Phe Conversion % (ee%)
OEt	NR	NR	33 (50 *S*)	77 (99 *S*)	100 (94 *R*)
OAllyl	NR	NR	24 (71 *S*)	87 (99 *S*)	100 (85 *R*)
OAc	NR	NR	32 (79 *S*)	91 (99 *S*)	100 (68 *R*)

NR=no reaction.
[a] Conversion monitored by GC.
[b] ee measured by chiral-phase GC.

a significant scale-up, Furstoss and co-workers[152,153] searched for effective ways to minimize typical problems of biotransformations: toxic effects of substrate and/or product and substrate solubility. The fundamental idea was to use an appropriate resin capable of both substrate feeding and product removal in situ. In the past, the problem of toxicity and solubility of a substrate was routinely treated by adding cyclodextrin to the biotransformation mixture. The new 'substrate feeding and product removal process' referred to as in situ SFPR was optimized for several parameters: the type of a reactor, substrate/product absorbent, pH, aeration, and the mode of substrate feeding. The best results were achieved with the bubble column reactor using macroporous resin (Optipore L-493). Under optimized conditions 25 g of racemic bicyclo[3.2.0]hept-2-en-60ne **1** were converted by *E. coli*/CHMO to products with volumetric productivity of 1.2 g/L/h. The two regiomeric lactones **2** and **3** were obtained with >98% ee.[153,154] Further improvements in the design of the reactor and careful optimization of all parameters permitted near-kilogram scale oxidation of ketone **1**.[155]

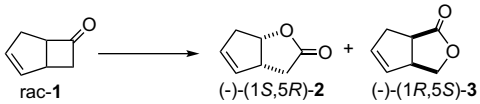


The 'substrate feeding and product removal process' (in situ SFPR) was applied to $CPMO_{Coma}$-catalyzed oxidation of 4-methylcyclohexanone and two other substrates. After optimization of the fermentation conditions the bioconversions were performed on 15 g scale in a desk top fermenter under growing and non-growing conditions. The reactions, carried out under non-growing conditions, were faster and gave better yields without compromising enantioselectivity. Thus, the combination of SFPR methodology with non-growing conditions was shown to be a more general and effective protocol for *E. coli*/BVMO-catalyzed multi-gram transformations.[156]

6.2. Isolated enzymes

6.2.1. Easy and practical PAMO (and its mutants)-catalyzed BV oxidations

To develop an alternative 'chemist-friendly' protocol for BVMO-catalyzed biotransformations Reetz and co-workers[157] chose as a model PAMO-catalyzed and PAMO mutants[129] catalyzed oxidation of a perennial favorite of BVMO substrates, *rac*-bicyclo[3.2.0]hept-2-en-6-one, **10**. Preliminary evaluation of the biocatalyst in the oxidation of **10** showed mutants to be very effective reagents (Table 11).



The preliminary evaluation of the biocatalysts in oxidation of substrate **1** gave excellent conversion for the three mutants (Table 11), however, increasing substrate concentration beyond 1 g/L resulted in dramatic decrease in conversion, probably due to substrate and/or product inhibition of the enzyme. A new protocol was developed and optimized to overcome these difficulties and facilitate PAMO-catalyzed transformations. Thermostable PAMO and its thermostable mutants are suitable candidates for the development as robust and practical bioreagents and the experimental conditions developed are indeed very simple and suitable for chemistry laboratories. Thus, the inhibition problem was solved by running the reaction in water/buffer and immiscible organic solvent. Secondary alcohol dehydrogenase (2°ADH) from thermostable *Thermoanaerobacter ethanolicus*[158] was used in NADPH regeneration. All enzymes, including ADH, were obtained from overexpressing *E. coli* strains and did not require purification; simple heat treatment was enough to purify them to near homogeneity. Under optimized conditions ketone **1** could be oxidized quantitatively in 24 h to the lactonic products in a concentration of 5 g/L. The same protocol was used successfully in kinetic resolution of *rac*-2-phenylcyclohexanone.[159]

In another study, PAMO and HAPMO oxidations of several sulfides were performed in a variety of aqueous/organic media. In general, the organic solvents decreased enzyme activity, although the magnitude of this effect depended on the type and concentration of the solvent. This negative effect was slightly offset by the enhanced solubility of sulfides in mixed solvent as compared to the aqueous buffers. A surprising discovery was that enantioselectivity of PAMO-catalyzed oxidation of several sulfides was significantly improved in buffer/methanol or ethanol media. In two cases, the methanol co-solvent caused reversal of PAMO's enantiopreference.[160] Similar effects of organic solvents on enantioselectivity of other enzymes had been noted before and in some cases a correlation with physico-chemical properties could be established.[161] In the case of PAMO it appears that short chain alcohols co-solvents may modify the character of the active site, possibly by hydrogen bonding to the enzyme's residues in or near active site.

6.2.2. Cofactor recycling using fusion protein

Since in some cases it is advantageous to use isolated enzymes for biotransformations, there is continuing interest in developing more efficient, less expensive, easier to use, or simply more elegant methods for cofactor recycling. Two recent reports are examples of imaginative progress in this area.

Fusion or chimeric proteins are made by joining two or more genes, which originally coded for separate proteins. A new advancement in cofactor recycling takes advantage of such fusion protein—reuniting a BVMO gene with a gene of soluble NADPH

Table 11
Preliminary PAMO and PAMO-mutants Baeyer–Villiger oxidation of **10**, performed at low concentration of substrate[157]

Enzyme	Conversion 24 h (%)	Lactone **2** ee (%)	Lactone **3** ee (%)
WT PAMO	43	92 (−)	48 (+)
P1-PAMO[a]	>95	80 (−)	99 (−)
P2-PAMO[a]	>95	93 (−)	>99 (−)
P3-PAMO[a]	>95	92 (−)	>99 (−)

[a] PAMO's mutant.[129]

regenerating phosphite dehydrogenase (PTDH).[162] As a 'free' protein PTDH catalyzes selectively oxidation of phosphite to phosphate with the concomitant reduction of NAD^+ to NADH. PTDH can also use $NADP^+$ as the oxidant, although less effectively.[163] The fusion proteins engineered from CHMO, CPMO, and PAMO preserve activity of both proteins: they catalyze Baeyer–Villiger oxidations and use inexpensive phosphite as electron donor for recycling of $NADP^+$ to NADPH. Tested in oxidations of several substrates, they produced results very similar to the parent biocatalysts. In a preparative-scale biotransformation, a crude cell extract from *E. coli* expressing CHMO/PTDH was used to convert 4-methylcyclohexanone into the corresponding lactone without need for the addition of $NADP^+$. This indicates that the amount of nicotinamide liberated during cell breakage is adequate for catalysis and that the regeneration system is functioning efficiently. The construction and development of new self-sufficient BVMOs promise to be a very important addition to the family of redox bioreagents.

6.2.3. Light-driven cofactor recycling

In a very different strategy, Reetz and co-workers[164,165] devised a catalytic scheme in which NADPH regeneration is omitted entirely and the reduction of flavin is powered by light with ethylenediaminetetraacetate (EDTA) as a sacrificial electron donor, Figure 21. In a preliminary study, PAMO-P3 mutant, purified by affinity chromatography,[129] was used as biocatalyst and ketones **10**, 2-phenylcyclohexanone, and 2-benzylcyclohexanone were the test substrates. Even under non-optimized conditions, the enzyme was stable for several hours and its selectivity was unaffected by unnatural regeneration conditions (Table 12).

The catalytic performance of PAMO, however, was significantly lower compared to an enzyme-coupled NADPH regeneration system. The under performance of light-catalyzed transformation was linked to the uncoupling of light-driven flavin reduction [166] and sterically restricted access of the mediator to PAMO-bound FAD.[165] When the light-driven regeneration approach was applied to flavin-dependent reductase, the Old Yellow Enzyme homologue YqjM as a model system, a higher catalytic turnover was obtained, apparently due to better access to the prosthetic group and the absence of oxidative uncoupling.[165]

The light-driven methodology is still under development; once perfected it should be applicable to other flavin-dependent BVMOs (providing that conditions can be adapted to accommodate BVMOs less stable than PAMO) and, as demonstrated in the case of YqiM reductase, to other flavin-dependent enzymes.

6.3. Synthetic applications

As is the case in the development of traditional chemical reagents, the development of Baeyer–Villiger (and other) bioreagents has been carried out with future synthetic applications in mind. The high enantio- and regioselectivity, the 'green' advantage, and the fact that *E. coli*/BVMO reagents can be grown and propagated makes them particularly valuable tools in the hands of chemists. With a large collection of the BVMO overexpression systems now available, a variety of enantiopure lactones and esters, which can be

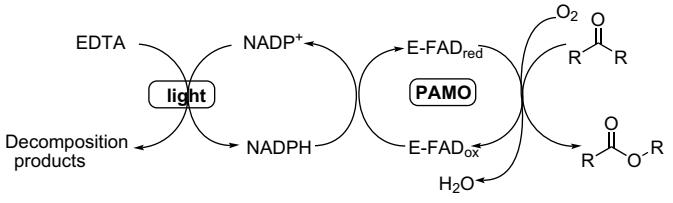
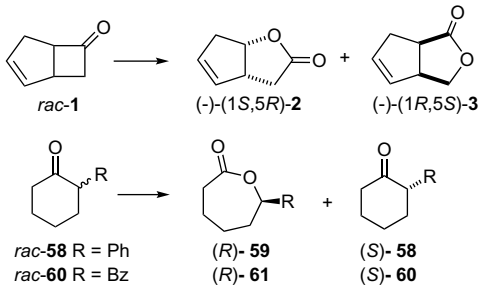


Figure 21. Light-driven regeneration approach system for PAMO.

Table 12
PAMO-P3[a] catalyzed Baeyer–Villiger oxidation performed under light[b] driven recycling of FAD



Substrate	Conversion (%)	Product lactone(s) ee (%)
rac-**10**	93 (95)[c]	>95 (**2**), 92 (**3**) [99 (**2**), 92 (**3**)]
rac-**58**	48 (<50)	97 (96)
rac-**60**	30 (40)	97 (99)

[a] PAMO's mutant.[129]
[b] Osram white light bulb (100 W).
[c] Values in parentheses are from the process using NADPH recycling.[129]

obtained via the biotransformation routes is rapidly growing. Many lactones are the key intermediates in the synthesis of important bioactive compounds and are therefore highly desirable fine chemicals. Since this point has been repeatedly stressed in reviews,[34,35] and in numerous individual publications featured in this report, only a few representative examples are shown here.

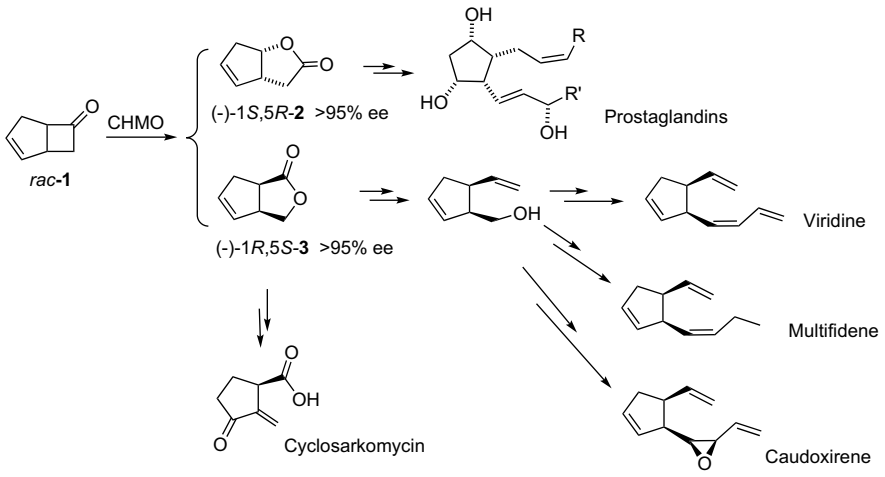
6.3.1. Bicyclic lactones

When BVMO-catalyzed oxidation of bicyclic ketone **1** is highly selective it gives a mixture of two enantiopure regioisomers: a 'normal' (oxygen insertion on the more substituted side) lactone **2** and an 'abnormal' lactone **3**. Equipped with two stereogenic centers and two functional groups that can be readily transformed, lactones **2** and **3** are excellent intermediates for asymmetric synthesis (Scheme 8). For example, (1*S*,5*R*)-**2** is a key element in the synthesis of prostaglandins,[167] while the 'abnormal' lactone (1*R*,5*S*)-**3** was used to prepare the brown algae, pheromones[168] and an antitumor agent cyclosarkomycin.[169] Today, enantiopure lactones **2** and **3** can be produced through biooxidation of a racemic ketone **1** on a kilogram scale by several of the available BVMO bioreagents.[152,153] It is noteworthy that their antipodes (1*R*,5*S*-**2** and 1*S*,5*R*-**3**) are also accessible via biooxidation of ketone **1** with the NADH-dependent diketocamphane monooxygenase (2,5-DKCMO) from (+)-camphor-grown *P. putida* NCIMB 10007 [(1*R*,5*S*)-**2**, 50% ee and (1*S*,5*R*)-**3**, >95% ee].[170]

6.3.2. γ-Butyrolactones

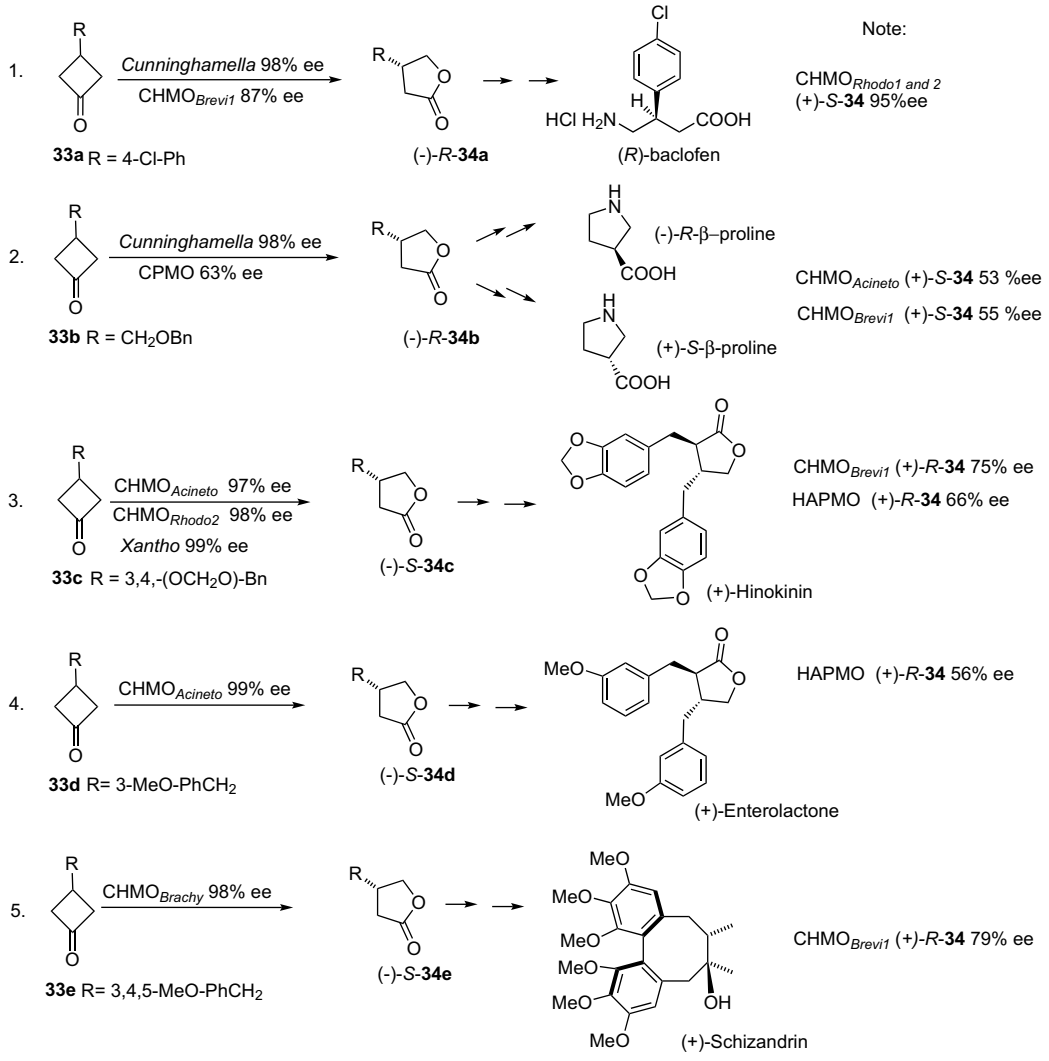
Optically pure enantiomers of the 3-substituted γ-butyrolactones have played an important role in the synthesis of a variety of natural products and therapeutic agents.[171] A few target compounds that employed γ-lactones as intermediates in their synthesis are shown in Scheme 9.

Baclofen, an agonist of the γ-aminobutyric acid GABA, was synthesized from prochiral 3-*p*-chlorophenyl-cyclobutanone **33a**. The required chirality was introduced via biological Baeyer–Villiger oxidation performed with fungus *Cunninghamella echinulata*.[172,173] Under optimized conditions γ-butyrolactone **34a** (R=4-Cl-phenyl) was obtained in 71% yield and (−)-*R* 98% ee.[174] Today, both enantiomers of this butyrolactone can be prepared: (+)-*S* 95% ee from *E. coli*/$CHMO_{Rhodo1}$-catalyzed transformation of **33a** and (−) *R* (87% ee) from *E. coli*/$CHMO_{Brevi1}$ reaction.[86d]



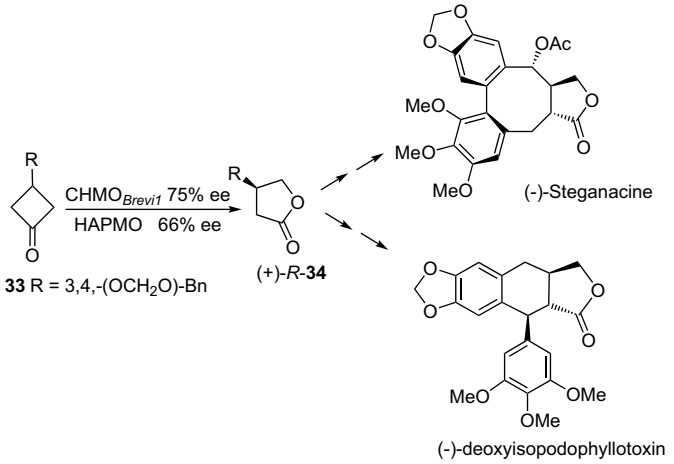
Scheme 8.

γ-Butyrolactone **34b** can be used in the synthesis of β-amino acids such as β-proline.[175] Highly enantioselective Baeyer–Villiger oxidation of **33** to (*R*)-**34b** (ee>97%) can be achieved again with the filamentous fungus *C. echinulata* NRRL 3655.[176] Although several BVMOs (CHMOs, CPMO, and HAPMO) all accept and oxidize these substrates, none of those currently available can match enantioselectivity of *C. echinulata*.[86d] The enantiomer (*S*)-**34** is available from the CHMO oxidation of **33**, but enantiomeric excess is low (55% ee).



Scheme 9.

Both antipodes of **34c**, **34d**, and **34e** are useful intermediates in the synthesis of various lignans, a class of biologically important compounds known for their antileukemic, antiviral, antifungal, and antineoplastic activities.[177] The two enantiomers can be prepared in excellent yields and with good to high enantioselectivities, as summarized in Scheme 10. Interestingly, in the case of the cyclobutanones **33c–e** substituted with piperonyl and 3-MeO-Bn groups, the corresponding (−)-*S*-**34c–e** lactones were obtained with higher enantiopurity in the oxidation catalyzed by $CHMO_{Acineto}$ than with *C. echinulata*.[176] The access to the (+)-*R*-**34c**,**d** enantiomers is via transformations with $CHMO_{Brevi1}$ or HAPMO, but only with modest enantioselectivity values (Scheme 10).



Scheme 10.

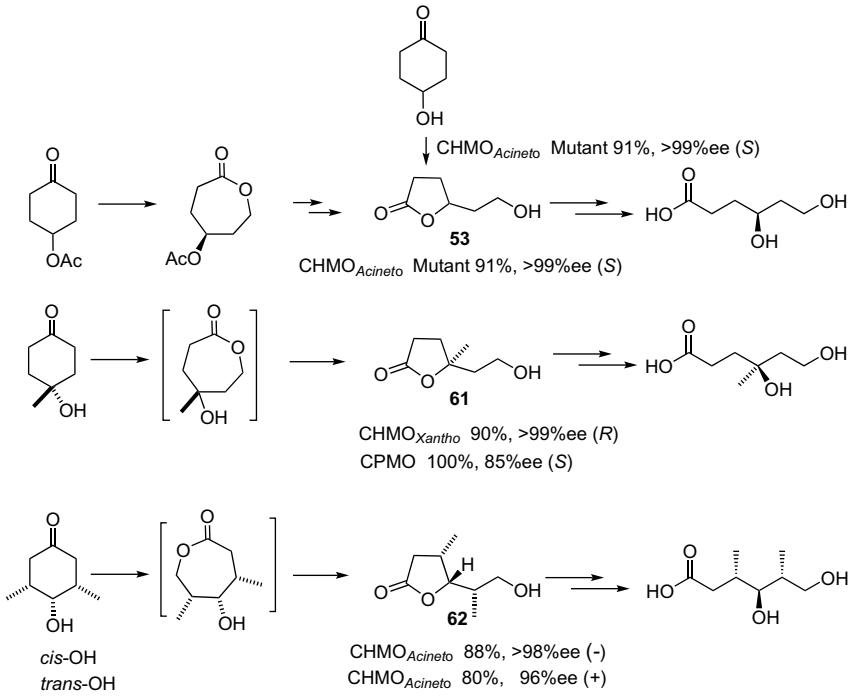
4-Hydroxycyclohexanones are oxidized to corresponding caprolactones, which spontaneously rearrange to γ-butyrolactones (Scheme 11). Fragments corresponding to lactones such as **53**, **62**, and **63** are found in great many natural products. For example, **53** represents 1,3 and 1,4 diol synthons. The lactones with two or more chiral centers next to each other (as in **62**) have several possible diastereomeric combinations, most of which are represented in natural products. These compounds, therefore, are versatile chiral synthons and their availability in optically pure form is of considerable synthetic interest. Lactone **62**, for example, was a key intermediate in the synthesis of a potent inhibitor of ribonucleic acid polymerase,[178] tirandamycin,[35,179] and can serve as precursor in the synthesis of a serine/threonine phosphatase inhibitor, calyculin A.[35,180] Biotransformations with BVMOS allow many such lactones to be prepared in an enantiopure form in one step, as shown in Scheme 11.[151,87a]

6.3.3. *Heterobicyclic lactones*

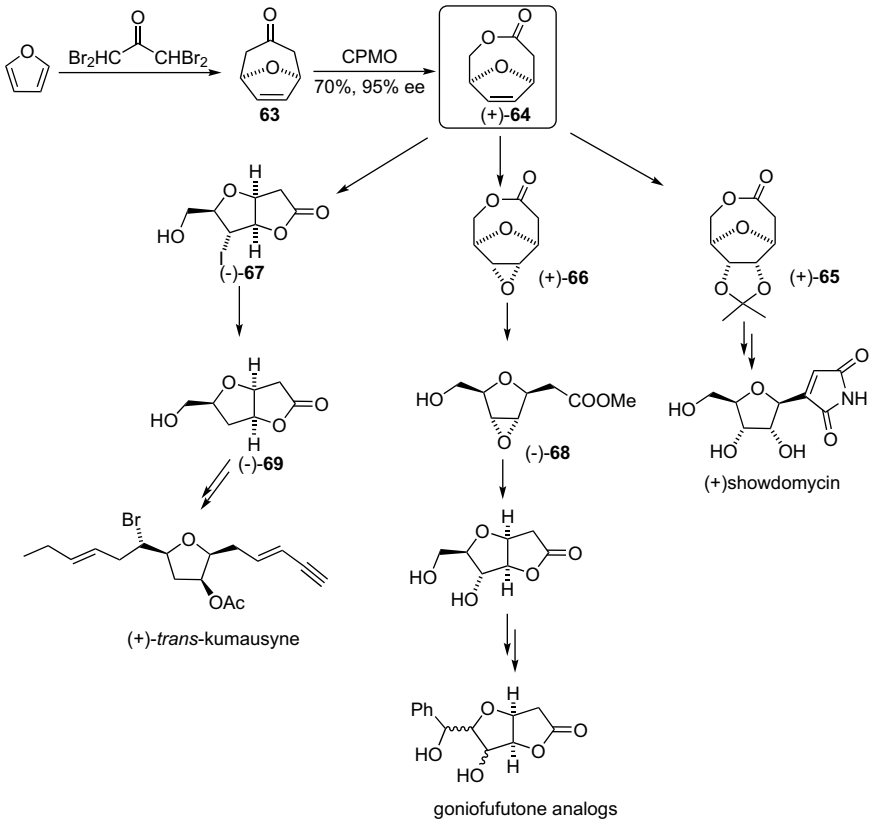
Bicyclic ketone **63** prepared as shown in Scheme 12 is converted to lactone **64** in good yield and high enantiopurity through CPMO-catalyzed Baeyer–Villiger reaction.[181] This lactone is a versatile starting material for the synthesis of several bioactive compounds. For example, it can be converted to the antibiotic (+)-showdomycin, a halogenated secondary metabolite of red algae (+)-*trans*-kumausyne, or goniofufurone analogs, which are cytotoxic to human tumor cells.[181]

6.3.4. *δ-Valerolactones*

CPMO-catalyzed Baeyer–Villiger oxidation of cyclopentanones to δ-valerolactones can accommodate a variety of alkyl and functionalized chains. These and other substituted lactones, as well as their ketone precursors, are often endowed with attractive odors and are used in perfumes and fragrances. Frequently, the two enantiomers of the same compound have very different smells and threshold values.[182] The growing interest in chirality–odor relationship, and the importance of these compounds in the fragrance industry encouraged search for effective ways to prepare them. As with butyrolactones and caprolactones, δ-valerolastones, particularly those substituted with functionalized side chains, are important building blocks for synthesis. Lactone (*S*)-**70**, corresponding to 1,2 and 1,5 diols, was used by Corey and co-workers in the total synthesis of leucotriene B5[183] Scheme 13, while its



Scheme 11.



Scheme 12.

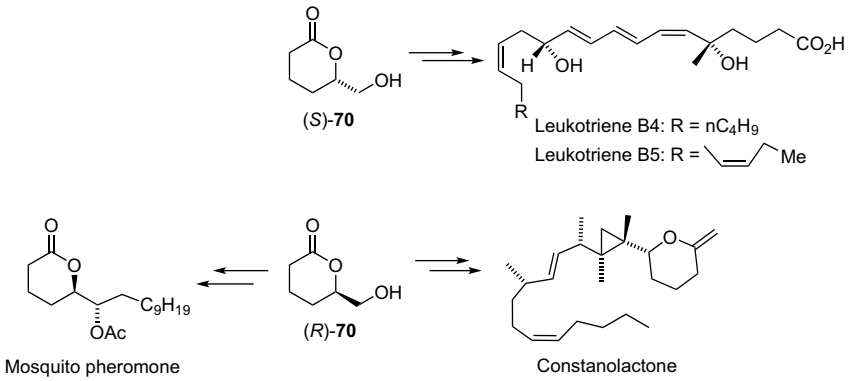
antipode provides structural characteristics suitable for the synthesis of compounds such as constanolactone[184] and mosquito oviposition pheromone (MOP).[185]

A variety of enantiopure or enantioenriched δ-valerolactones can be prepared through biooxidations of cyclopentanones. Although both CPMO and CHMO readily convert cyclopentanones, the CHMO-catalyzed resolution of 2-substituted cyclopentanones is considerably more selective; see Scheme 14.[29,79] The dynamic kinetic resolution of 2-benzyloxymethylcyclopentanone with *E. coli*/CHMO, discussed earlier (Fig. 3) gave (*R*)-lactone in 85% yield with 97% ee.[62] Thus, in other cases where dynamic kinetic resolution can be applied, good yields and high enantioselectivity are achievable.

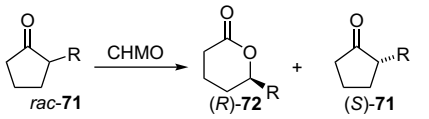
A number of recently discovered BVMO enzymes need more extensive screening to identify the full extent of their substrate base and selectivity. With the more complete profiles of these enzymes and their mutants new biocatalytic routes to other important building blocks for organic synthesis will surface. Biocatalysis with BVMOs is still very much work in progress.

7. Conclusions

CHMO was isolated and purified in 1976; its overexpressions in *S. cerevisiae* and, particularly, in *E. coli* over two decades later gave rise to the first 'designer bioreagents.' In the following few years *E. coli* expression systems for a growing number of Baeyer-Villigerases multiplied and so did the synthetic potential of these bioreagents. Now, a sizeable collection of available BVMO expression systems can be used to convert many ketones to enantiopure or enantioenriched lactones or esters, and to oxidize many sulfides to enantiopure sulfoxides. At present, there is no method, which can



Scheme 13.



R = n-Hexyl	32%; 98% ee 42%; 98% ee
R = $(CH_2)_3Br$	47%;95% ee 47%; 92% ee
R = $CH_2OAllyl$	33%;95% ee 19%; 81% ee
R = CH_2OBn	43%; 97% ee 48%: 43% ee
R = CH_2OBn(DKR)	85%;97% ee

Scheme 14.

rival Baeyer–Villiger biooxidations with respect to stereoselectivity and substrate base. Indeed, Baeyer-Villigarases are excellent reagents for organic synthesis and the simplest way for non-specialists to use them is by employing whole-cell *E. coli* overexpression of the chosen protein.

In principle, isolated enzyme-catalyzed reactions can be performed at higher substrate concentrations and make downstream processing easier. However, even though progress in the use of isolated enzymes is impressive and finds extensive industrial applications, at present time the 'designer microorganism' reagents are more chemist friendly.

A frequently voiced critique of whole-cell methodology is that the volume yield of the product(s) is low and many organic substrates have limited solubility in water. As discussed earlier, these problems are being solved. The use of non-growing cells, which perform the reactions in aqueous/organic media, and optimization of reaction conditions can minimize many difficulties, while resin-based in situ substrate feeding and product removal allows the CHMO-catalyzed Baeyer–Villiger oxidation of racemic ketone **1** to produce kilogram quantities of enantiopure regiomeric lactones **2** and **3** in a 50 L reactor.

Recent studies of the whole-cell *E. coli*/BVMO-catalyzed biotransformation in a microtiter plate showed that the performance of individual biocatalyst with each substrate in individual wells could be monitored, and that fermentation and bioconversion stages were reproducible.[186] A 24-well microtiter plate screening of several *E. coli*/CHMO mutants against a library of structurally diverse ketones rapidly identified the best mutant/substrate match.[187] Commercial availability of such libraries of BVMO (and other biocatalysts of synthetic value) will allow chemists to quickly discover the best bioreagent for his substrate. Preparative-scale protocols are simple and optimization of biotransformation conditions is not more onerous than optimization of classical chemical reactions. BVMOs expressed in *E. coli* can be stored *and* propagated, an accomplishment that is not matched by chemical reagents. Finally, the ultimate benefit of bioreagents is their products; chemo-, regio-, and enantiopure products, sometimes with several stereogenic centers that are generated in a single step process.

The overexpression of BVMOs in *E. coli* provided access to large quantities of these proteins for in-cell transformations and isolation (and purification). The latter made easier research and applications involving isolated enzymes. The solution of the crystal structure of phenylacetone monooxygenase from *T. fusca* (PAMO)[109] and the most recent kinetic studies of PAMO's catalytic mechanism[188] lead to a better understanding of PAMO's (and other members of BVMO family) active site characteristics, substrate acceptance, and selectivity (or lack of it). The grasp of the factors, which make enzymes regio- and enantioselective, derived from continuing structural,[189] and kinetic studies, as well as from directed evolution and rational design[190] experiments, will make possible development of better bioreagents and will inspire chemists to design better asymmetric organo- and organometallic catalysts. In the meantime, it made the already available bioreagents[191] 'a 'true' chemical reagent: predictable, general, and reproducible.'

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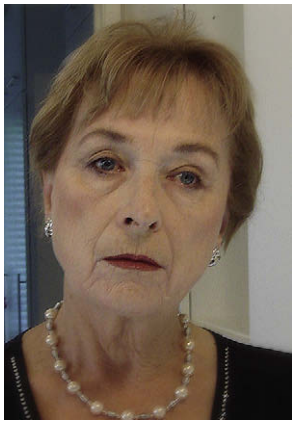
Biographical sketch



Margaret M. Kayser is Professor Emerita at the University of New Brunswick. She obtained B.Sc. and Ph.D. degrees from the University of Ottawa where she worked with Peter Morand. After additional research experience at the Laboratoire de Chimie Théorique, Université de Paris-Sud, Centre d'Orsay (Nguyen Ahn) and the Institut de Chimie Moléculaire, Université de Strasbourg (Jean-Marie Lehn), she taught at the Université de Moncton, Mount Allison University and, for 20 years at the University of New Brunswick in Saint John. Her initial interests in reaction mechanisms, although never entirely abandoned, gave way to a passion for biocatalysts and biocatalysis.