Mapping of the Functional Active Site of Baeyer–Villigerases by Substrate Engineering

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An active site model for enzyme catalysed Baeyer–Villiger reactions is proposed and validated by transformation of the tricyclic ketone **6**; Baeyer–Villiger reactions (and Baeyer–Villigerases) are classified by the stereochemistry of the hydroxyperoxide intermediate.

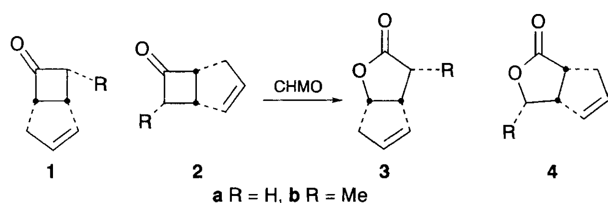
The enantiomeric bicyclic ketones **1** and **2** undergo Baeyer–Villiger ring expansion by the cyclohexanone monooxygenase (CHMO) from *Acinetobacter sp.* (NCIMB 9871) to give the regioisomeric lactones **3** and **4** of high enantiomeric purity (Scheme 1).[1] The amino acid sequence of the enzyme shows no evidence for multiple active sites, therefore the two regioisomers are likely to be formed at the same active site by two modes of binding.

The mechanism of the CHMO catalysed Baeyer–Villiger ring expansion proceeds by essentially the same mechanism as the abiotic reaction, except that a reduced flavin hydroperoxide is utilised instead of a peracid (or other hydroperoxide). A major tenet of the currently accepted mechanism[2] is that the migrating bond lies antiperiplanar to the oxygen–oxygen bond of the peroxide. Moreover, it is well known that electronically unbiased bicyclo[3.2.0]heptan-6-ones react with nucleophiles predominantly on the *exo* face.[3] The geometry of the transition state and the diastereofacial selectivity of nucleophilic attack therefore suggest that the enantiomeric ketones **1** and **2** are bound as hydroxyperoxides† at the active site of CHMO such that the cyclobutanone rings are in overlapped positions and the cyclopentene rings lie at approximately 90° to each other (Fig. 1).[4]

This model was tested by preparing and biotransforming the prochiral tricyclic ketone **6**, which is a composite of the structural elements of the bicyclic ketones **1** and **2**.[5] Treatment of cyclopentadiene and 4-chlorobutyryl chloride with triethylamine in hexane gave the known bicyclic ketone **5a**.[6] The precedents for the planned intramolecular free radical addition did not augur success. Berge and Roberts reported that the chloroethyl alkene **5a** is reduced by tri-*n*-butyltin hydride to the ethyl alkene **5b**[7] and Dowd and Zhang have shown, in a plethora of closely related systems, that many other modes of reaction are accessible for the radical intermediate.[8] In the event, slow addition of tri-*n*-butyl tin hydride to the chloroalkene **5a** in THF gave the symmetrical tricyclic ketone **6** (75%), which had only six signals in the ^{13}C NMR spectrum. The *endo*-ethyl bicyclic ketone **5b** (10–15%) was also isolated when the reaction was run in benzene or toluene. The tricyclic ketone **6** was readily oxidised to the corresponding racemic lactone **7** by hydrogen peroxide–acetic acid. Alternatively, the lactone **7** was prepared by inverting the sequence of steps. Baeyer–Villiger oxidation of the chloroethyl ketone **5a** with hydrogen peroxide–acetic acid gave a mixture (70:30) of the lactones **8** and **9**, both of which underwent free radical cyclisation to the lactone **7** without concomitant reduction. The structure assignments for the chloroethyl lactones **8** and **9** were confirmed by treatment with DBU to give the cyclopropane **10** and untransformed lactone **8**.[6]

Ring expansion of the tricyclic ketone **6** with a number of organisms was enantioselective (Table 1)‡ and this selectivity was increased when purified enzyme was used.

Similarly, whole cell biotransformation by *Acinetobacter sp.* NCIMB 9871 of the *endo*-chloroethyl ketone **5a** gave a mixture of the lactones **8** and **9** (27:73; 82%, 97% e.e., respectively). *Xanthobacter* DSM 431 whole cell conversion of the same ketone **5a** gave a similar mixture of the lactones **8** and **9** (27:73), but both were enantiomerically pure (>99% e.e.). In both cases all of the ketone **5a** was consumed and hence some material must have been lost to catabolic processes. The mixture of lactones **8** and **9** from the *Acinetobacter sp.* NCIMB 9871 whole cell biotransformation underwent tin hydride mediated



Scheme 1

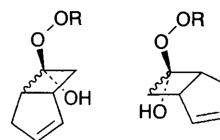
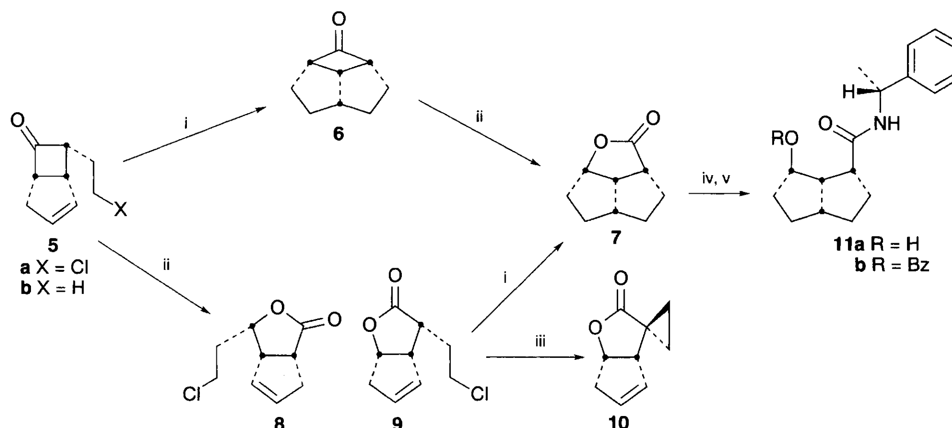


Fig. 1 The migrating bond is shown as a wavy line



Scheme 2 *Reagents and conditions*: i, Bu_3SnH, THF, AIBN, reflux; ii, H_2O_2, acetic acid, water or Baeyer–Villigerase; iii, DBU, CH_2Cl_2; iv, BuLi, (*R*)-α-methylbenzylamine; v, BzCl, py

cyclisation to give the same enantiomer of the lactone **7** (92.7% e.e.),§ as that produced by the same organism from the tricyclic ketone **6**.

The absolute stereochemistry of the lactone **7** was established by X-ray crystal structure determination of the derivative **11b**. Treatment with (*R*)-α-methylbenzylamine even under reflux at 220 °C failed to open the lactone ring; however this was achieved with the lithium salt of the amine. The amide **11a** so-formed was benzoylated to give the ester **11b** which was shown to have the absolute configuration indicated and as predicted by the superimposition model.

Alphand and Furstoss have proposed a model for Baeyer–Villigerases in which the active site discriminates groups of differing sizes.[9] In contrast the results we present here and analysis of prior work indicates that at least for the small and medium ring mono-, bi- and tri-cyclic ketones investigated thus far, the configuration of the hydroxyperoxide intermediates (Fig. 1), modulated by the diastereoselectivity of addition of the hydroperoxide, controls the stereoselectivity of the reaction. This is equivalent to asserting that a particular Baeyer–Villigerase will only be capable of stabilising one hydroxyperoxide configuration (Fig. 2). The enantioselectivity of a given reaction can then be predicted from the diastereofacial selectivity of addition of the peroxide to the ketone substrate.[10] This paradigm is easily applied and should have considerable merit for comparing stereoselectivities.

Table 1 Biotransformation of the tricyclic ketone **6** to the lactone **7**

Organism	e.e. (%)[a]
Acinetobacter sp. NCIMB 9871	87.5[b]
Acinetobacter sp. NCIMB 9871, purified enzyme	>98
Xanthobacter autotrophicus DSM 431	87.5
Pseudomonas putida NCIMB 10007 (+)-camphor[c]	−5.5[e]
P. putida NCIMB 10007 (−)-camphor[c]	56.5
P. putida NCIMB 10007 (+)-camphor,[c] purified enzyme (MO1)[d]	>−98[e]

[a] Enantiomeric excess of the slowest eluting analyte by GC. [b] 75.4% conversion, all others 100% conversion. [c] Substrate on which the organism was grown. [d] This transformation was run by P. W. H. Wan, A. J. Willetts and S. M. Roberts at the University of Exeter. [e] Expressed relative to the other enantiomer.

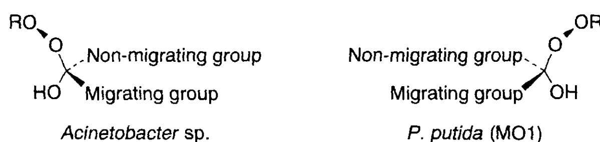


Fig. 2

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Footnotes

† The intermediates in the Baeyer–Villiger ring expansion are shown as hydroxyperoxides for convenience. We do not exclude the possibility that some other electrophile may react with the incipient alkoxide.

‡ Enantiomeric excesses determined by chiral GC on Lipodex D (Macherey-Nagel, 50 m) are reproducible within ±0.3%. Temperature programmes and retention times/min: isothermal 170 °C **7** 18.1, 18.9; isothermal 160 °C **9** 54.3, 56.4; **8** 67.1, 69.1.

§ The calculated e.e. of the tricyclic lactone **7** is 92.9%, based on the ratio of the chloroethyllactones **8** and **9** and their e.e.s and with the assumption that they are from opposite enantiomeric series.

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