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Effect of substrate concentration on the enantioselectivity of cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* and its rationalization

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Abstract

The ee values of lactone **3**, and not of lactone **2**, obtained from the enantiodivergent oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one **1**, catalyzed by cyclohexanone monooxygenase from *Acinetobacter calcoaceticus*, were found to be markedly dependent on the degree of conversion and substrate concentration. The results are rationalized on the basis of a model which hypothesizes the binding of a second substrate molecule to an enzyme site distinct from the catalytic site. © 2000 Elsevier Science Ltd. All rights reserved.

Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CYMO) is a flavoenzyme able to catalyze the nucleophilic oxygenation of aldehydes and ketones (Baeyer–Villiger oxidation), as well as the electrophilic oxygenation of various heteroatoms such as sulphur, selenium, nitrogen, boron and phosphorus.[1–5] What makes CYMO attractive for synthetic purposes is the fact that both types of reaction can proceed with exquisite selectivity with a wide variety of natural and synthetic substrates. Thus, the enzyme (as whole-cell preparation, partially purified protein or pure enzyme) has been applied as a chiral catalyst for the transformation of countless racemic and prochiral ketones to chiral lactones (for a review see Ref. 5) and organic sulphur compounds to optically active sulphoxides (for a review see Ref. 3).

One of the most interesting transformations catalyzed by CYMO is the Baeyer–Villiger oxidation, first described by Furstoss and coworkers,[6,7] of bicyclo[3.2.0]hept-2-en-6-one **1** (which subsequently also became a sort of reference substrate for other monooxygenases) into the two regioisomeric lactones **2** and **3** (Scheme 1), which are chiral synthons particularly useful in the synthesis of prostaglandins and nucleosides.

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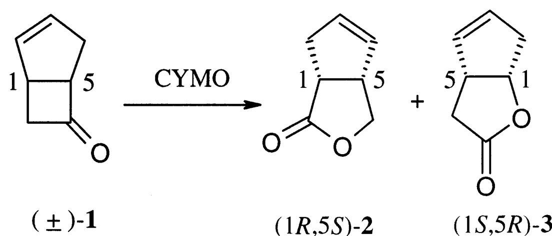

Scheme 1. CYMO-catalyzed enantiodivergent oxidation of racemic bicyclo[3.2.0]hept-2-en-6-one **1** to lactones (1*R*,5*S*)-**2** and (1*S*,5*R*)-**3**

In the framework of continuing research on synthetic applications of monooxygenases for both heteroatoms[3,8,9] and Baeyer–Villiger oxidation,[10] we reinvestigated the time course of the CYMO[11]-catalyzed oxidation of **1**.

Contrary to what had been previously reported by Furstoss and coworkers,[7] who observed a constant 1:1 ratio between **2** and **3**, we found to our surprise that this ratio changed with time (with regioisomer **3** being formed much faster than **2**) and approached a 1:1 value only at complete conversion (Fig. 1). Furthermore, whereas the ee of lactone **2** (formed by the so-called 'abnormal' oxygen insertion between the less substituted carbon atom and carbonyl group of **1**) was very high (ee ≥ 98%) and remained constant through the entire reaction period, the ee of lactone **3** (formed by 'normal' oxygen insertion) increased as a function of time from an initial 27% value to a final value of 83%, when the conversion was complete. It should also be noted that the enantiomeric excess of lactone **3** (ee 83%) was substantially lower than previously reported (ee 98%).[6]

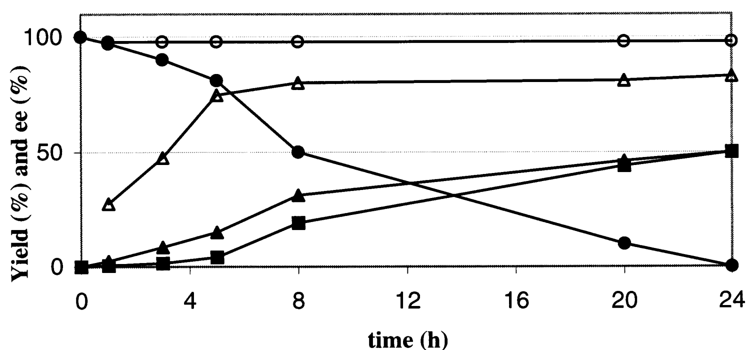

Figure 1. Time course of CYMO-catalyzed oxidation of **1** to lactones **2** and **3**. Compound **1** (92 mM) (≥98% pure, provided by Fluka) was dissolved in 10 mL of 0.05 M Tris–HCl buffer, pH 8.6, containing 0.5 mM NADP, 1 M 2-propanol, 50 units of CYMO and 200 units of alcohol dehydrogenase from *Thermoanaerobium brokii* for coenzyme regeneration. The degree of conversion and the enantiomeric excess of the products were determined on ethyl acetate extracts by chiral GC with a CP-cyclodextrin column (50 m, 0.25 mm ID, Chrompack) at 130°C with H_2 as a carrier gas. Retention times were: (1*R*,5*S*)-**1**, 4.758 min; (1*S*,5*R*)-**1**, 4.799 min; (1*R*,5*S*)-**2**, 17.40 min; (1*S*,5*R*)-**2**, 17.60 min; (1*R*,5*S*)-**3**, 16.63 min; (1*S*,5*R*)-**3**, 17.28 min. (●) Percentage of remaining substrate **1**; (■) percentage of formed lactone **2**; (▲) percentage of formed lactone **3**; (○) (%) ee of lactone **2**; (△) (%) ee of lactone **3**

As a subsequent step, the effect of substrate concentration, investigated in the 4.6–92 mM range, on the time course of formation and enantiomeric excess of lactones **2** and **3** was studied. It was found that **3** was formed faster than **2** in all cases, independent of substrate concentration. Furthermore, whereas the enantiomeric excess of the 'abnormal' lactone **2** remained

constant and high, that of the 'normal' lactone **3**, determined at complete substrate conversion, increased as the substrate concentration decreased (Table 1). Thus, with 92 mM substrate the ee of **3** was 83%, whereas with 4.6 mM substrate the ee increased to 98%, a value that is in good agreement with the data reported by Furstoss and coworkers (ee 98%),[6] who carried out the conversion at approximately 9 mM substrate concentration.

Table 1
Effect of substrate concentration on the ee of the produced lactones (1*R*,5*S*)-**2** and (1*S*,5*R*)-**3**, in the CYMO-catalyzed oxidation of **1**[a]

Substrate concentration (mM)	Ee (%)	
	2	**3**
4.6	≥98	≥98
7.4	≥98	95
12.0	≥98	91
23.0	≥98	87
46.0	≥98	85
92.0	≥98	83

[a] The ee values refer to the data obtained at complete conversion of substrate into products.

The conversion experiments carried out at different substrate concentrations revealed that CYMO underwent marked substrate inhibition. This might be the reason for the anomalous behaviour of the time course of the substrate conversion at high concentrations of **1** (92 mM), when a rate increase as a function of time (at least until 8 h) was observed (Fig. 1). This hypothesis is also supported by the finding that the behaviour became normal at substrate concentrations below 10 mM (data not shown). A spectrophotometric investigation[12] showed that the K_m of CYMO for substrate **1** was 1.7 μM, whereas the K_i for the substrate itself was 0.12 mM. From the study it appeared that the activity of CYMO at 92 mM substrate (that was the highest concentration checked) was only 10% of that obtained at 50 μM substrate, that was the concentration at which CYMO displayed the highest activity.

In order to explain the dependence of enzyme enantioselectivity on substrate concentration, a rationale based on the previously developed active site model for CYMO[13,14] is proposed (Fig. 2). Substrate **1** would bind not only to the catalytic site but also (though with much less affinity) to a nearby distinct site, affecting to different extents the different binding modes (and subsequent transformation) of the enantiomers of **1**. When the substrate binding to the second binding site is negligible, i.e. at low substrate concentration, the following behaviour might be envisaged: enantiomer (1*R*,5*S*)-**1** exclusively follows route a leading to (1*S*,5*R*)-**3**, since route a′ is prevented by severe steric hindrance. On the other hand, enantiomer (1*S*,5*R*)-**1** preferentially only follows route b, since steric hindrance does not appear so severe to completely prevent route b′. As a result, besides (1*R*,5*S*)-**2** a certain amount of (1*R*,5*S*)-**3** is also formed.

At increasing substrate concentrations, the binding of **1** to the second site would increase, with a progressive hindrance of route a and route b because of steric reasons; route a′ and route b′, on the other hand, would not be affected (Fig. 2). As a result, the higher the substrate concentration, the higher the proportion of (1*R*,5*S*)-**3** that will be formed. This will lower the ee of the predominant normal lactone (1*S*,5*R*)-**3** (see Table 1). As substrate oxidation proceeds, its concentration and inhibiting effect decrease and, thus, the ee of lactone **3** increases (Fig. 1). The

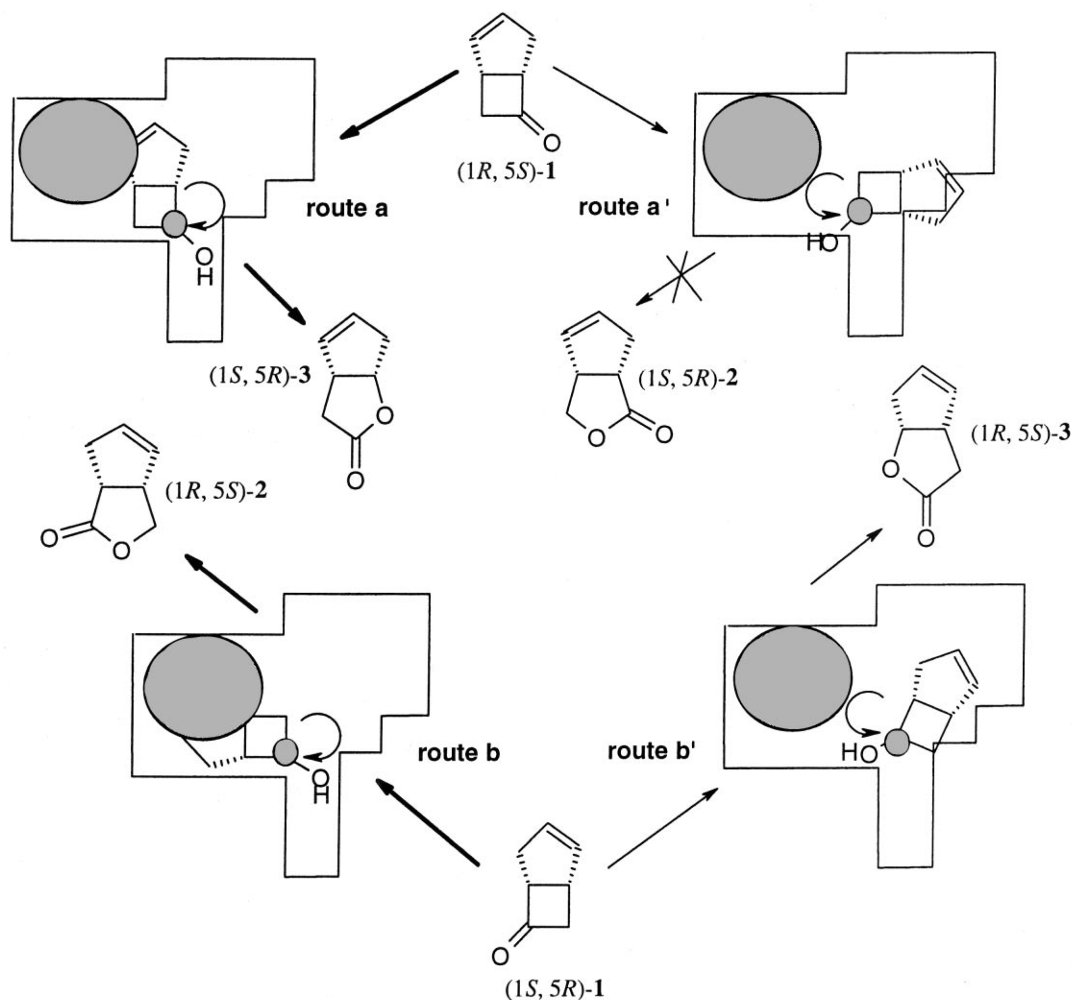

Figure 2. Top perspective view of the binding modes of substrate **1** in the active site model of CYMO. The small shaded circle represents the catalytically essential region (oxygen), whereas the large shaded circle represents the second binding site for **1**

oxidation of (1*R*,5*S*)-**1** instead would remain highly regioselective because, even though route a is hindered by the binding of the substrate at the second site, route a′ is, as stated before, completely prevented. Therefore, the ee of the predominant abnormal lactone (1*R*,5*S*)-**2** remains very high, independent of the degree of conversion (Fig. 1) and substrate concentration (Table 1). It should be emphasized that the existence of a regulatory site distinct from the catalytic site has already been hypothesized for some mammalian flavin-containing monooxygenases.[15]

The effect of substrate concentration on enantioselectivity, which has not been described before for either CYMO or other Baeyer–Villigerases, is of practical importance because of the remarkable synthetic potential of this class of enzymes. Work is in progress to see if the phenomenon occurs with other substrates and other monooxygenases.

Acknowledgements

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