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Exploring the Temperature Effect on Enantioselectivity of a Baeyer-Villiger Biooxidation by the 2,5-DKCMO Module: The SLM Approach

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Temperature is a crucial parameter for biological and chemical processes. Its effect on enzymatically catalysed reactions has been known for decades, and stereo- and enantiopreference are often temperature-dependent. For the first time, we present the temperature effect on the Baeyer-Villiger oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one by the type II Bayer-Villiger monooxygenase, 2,5-DKCMO. In the absence of a reductase and driven by the hydride-donation of a synthetic nicotinamide analogue, the clear trend for a decreasing enantioselectivity at higher temperatures was observed. "Traditional" approaches such as the determination of the enantiomeric ratio (*E*) appeared unsuitable due to the complexity of the system. To quantify the trend, we chose to use the 'Shape Language Modelling' (SLM), a tool that allows the reaction to be described at all points in a shape prescriptive manner. Thus, without knowing the equation of the reaction, the substrate *ee* can be estimated that at any conversion.

Introduction

Enantiomerically pure compounds are highly demanded as they serve as effective drugs or drug precursors.[1] The lightning progresses in molecular biology and biocatalysis have provided access to numerous established methods to synthesize enantiopure compounds from whole cells or purified enzymes.[2] Consequently, the stereochemical outcome of enzyme-catalysed reactions is of great interest in biocatalysis and its control of remarkable value.[1a,b,2b] Optimizing the (stereochemical) outcome, however, requires detailed knowledge of all reaction conditions, both biotic and abiotic factors.[3]

Temperature is one of the key parameters that affect enzymatic reactions.[3a,4] Although the operational temperature range of reactions catalysed by an isolated biocatalyst is relatively narrow compared with abiotically-catalysed reactions,[5] its effect on the stereo- and enantiopreference of enzymes[3a,4e,6] is a easy to control parameter.[4d,e,6c,7] The effect of temperature on the stereoselectivity of enzymes has been demonstrated for oxidoreductases, such as alcohol dehydrogenases (EC 1),[4b,c,8] lipases[9] or amidases[4d] (EC 3). This phenomenon is expected to be present to a higher extent but, because the effect might not be clearly visible, the number of examples remain low.[4b]

Recently, we demonstrated the independence of the oxygenase enzyme 2,5-diketocamphane-monooxygenase I (2,5-DKCMO), a rare type II Baeyer-Villiger monooxygenase (BVMO),[10] to catalyse the BV oxidation from any reductase auxiliary enzyme responsible for the reduction of the flavin cofactor FMN.[11] 2,5-DKCMO is the FMN-dependent monooxygenase of a two-component flavoprotein monooxygenase (FPMO) involved in the camphor degradation pathway of *Pseudomonas putida* ATCC 17453.[12]

In this study, we report the temperature effect on the enantioselectivity of 2,5-DKCMO module. The reaction was initiated by reduction of FMN by 1-(2-carbamoyl methyl)-1,4-dihydronicotinamide (AmNAH, caricotamide), a stable and water-soluble synthetic nicotinamide analogue. Due to the extremely narrow substrate specificity of the oxygenase,[12] it applied to *rac*-bicyclo[3.2.0]hept-2-en-6-one **1**, a ketone model substrate for BV oxidations,[13] exclusively (Scheme 1). The reaction was investigated at mediate temperatures (283 K to 303 K), at which we observed a clear shift of the enantioselectivity of the enzyme in the conversion of the racemic ketone with a greater preference for (−)-(1*S*,5*R*)-ketone at lower temperatures. To the best of our knowledge, this is the first report of the temperature effect on the selectivity of a BVMO-catalysed reaction, as well as on a two-component flavoprotein monooxygenase.

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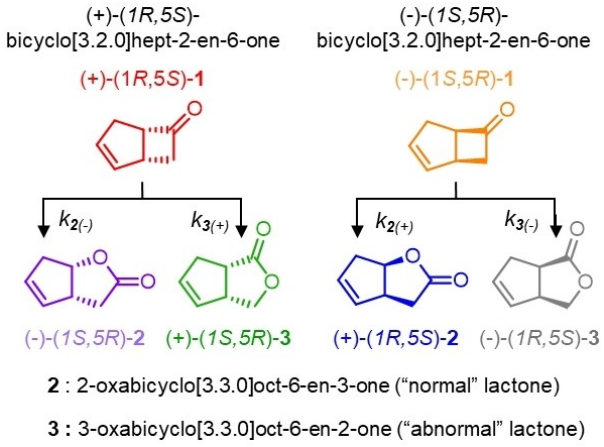
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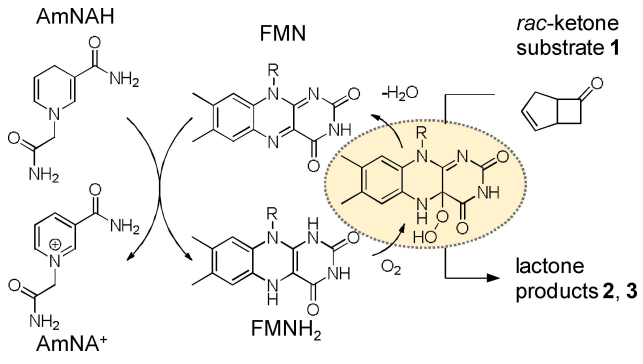


Scheme 1. Enantio- and regioselective enzymatic BV oxidation of *rac*-**1** with each enantiomer of the ketone as the precursor of two regioisomeric lactones **2** ("normal") or **3** ("abnormal"). Reaction rate order: $k_{2(+)} > k_{3(+)} > k_{2(-)} \gg k_{3(-)}$.

Results and Discussion

In this work, the FMN cofactor is chemically reduced by a direct hydride transfer *via* the artificial hydride donor AmNAH (Scheme 2).[11] The fully reduced flavin hydroquinone ($FMNH_2$), which is the electron mediator for the 2,5-DKCMO, diffuses into the active site of the enzyme, where the reactive flavin C4a-hydroperoxide is formed in the presence of molecular oxygen.

Compared with the 'native' system driven by the nicotinamide adenine dinucleotide cofactor (NAD(P)H) and a reductase, the AmNAH-driven reaction is a simpler approach. However, the (inter)actions taking place at the active site of the oxygenase are unknown, as the mechanistic model for the BV oxidation in two-component FPMO remains to be elucidated.[10d,14] The prediction of the mechanistic details appears to be particularly challenging for the present reaction, and a simple mechanism, as is required for most of the kinetic models, cannot be



Scheme 2. AmNAH-driven BV oxidation of *rac*-bicyclo[3.2.0]hept-2-en-6-one **1** by the 2,5-DKCMO. The enantioselective BV oxidation of the model ketone to form the corresponding lactones **2** (normal) and **3** (abnormal) takes place in the active site of the oxygenase in which the flavin C4a-hydroperoxide, the reactive species (highlighted in yellow) is formed. The (re)oxidized FMN diffuses in the reaction medium to be reduced by AmNAH.

proposed. A closer look at the time curve of each compound and its enantiomers (Figure S2) revealed a more complex behaviour than a classical simple kinetic resolution as previously described.[11] Thus, at lower temperatures, two reaction phases were visible. In the beginning, both ketone enantiomers were continuously consumed, with (−)-(1*S*,5*R*)-**1** at faster rates. Once the concentration of this enantiomer was negligible, an acceleration of the consumption of (+)-(1*R*,5*S*)-**1** was observed. Similar observations were previously described with epoxide hydrolases (EC3),[15] resulting from the vast difference in the K_M of the enantiomers. In our case, the simultaneous but not equivalent formation of both corresponding regioisomer lactones **2** and **3** increases the complexity even more.

Biotransformations were performed at different temperatures from 283 K to 303 K with 4 K-intervals. As expected, the activity (initial rate) increased with temperature, from 15 to 25 μM min^{-1} (see Figure S3, Supporting Information) and the enantiomeric excesses (*ee*) of the ketone increased also faster as reported Figure 1A. In Figure 1B, we plotted the ketone *ee* as a function of conversion, which allows us to cancel out the effect caused by the increase in activity, leaving only the impact of temperature on enantioselectivity visible. We observed a difference between the curves at the ends of the temperature range (lines with empty symbols). Thus, for the same conversion, the ketone *ee* values were higher at 283 K than at 303 K, indicating

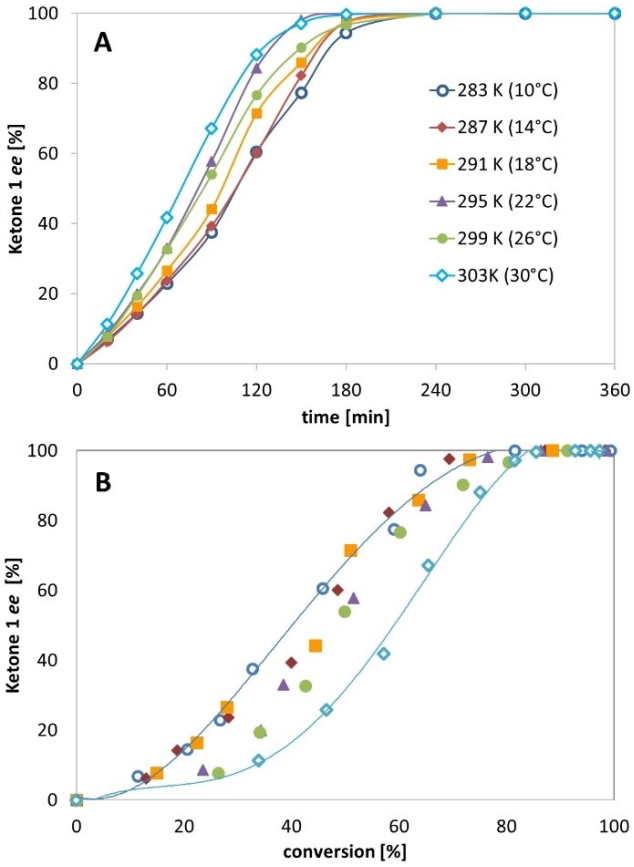


Figure 1. Enantioselective BV oxidation of *rac*-**1** by 2,5-DKCMO from 283 K to 303 K. Plots of substrate *ee* versus time in A, versus conversion in B.

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an increase in enantioselectivity. However, this effect was much less noticeable for intermediate temperatures (filled symbols).

Classically, such an effect can be quantified by the determination of the enantiomeric ratio *E* for each temperature. The *E*-value characterizes the ability of the enzyme to discriminate between two enantiomers and is defined by the formula:[16]

$$E = (k^R_{cat}/K^R_M)/(k^S_{cat}/K^S_M)$$

The usual way of calculating it, from the enantiomeric excesses of substrate and product, cannot be applied here as two regioisomeric lactones were formed. Therefore, we tentatively calculated *E* through the *ee* of the substrate and the conversion of the reaction (see Supporting Information). However, the theoretical curve, simulated from a nonlinear least square analysis, did not fit well with the experimental data, especially at higher temperatures (see Figure S6, Supporting Information). This observation further indicates the misfit with the (simple) standard models of kinetic resolutions as described by Sih.[17]

Despite the intricated reaction mechanism, we aimed to outline the effect of the temperature on the reaction system. A novel strategy, the Bayesian-like concept of 'Shape Language Modeling' (SLM, in MATLAB®) was applied to design a description for each of the investigated temperatures. SLM is a shape prescriptive approach, not a fixed mechanistic-mathematical model.[18] The method allowed us to avoid forcing the data into a model for the (uncertain) mechanism, which would be purely speculative. Instead, we describe the reaction in a prescriptive manner using sets of shape primitives for the curve fit. SLM permitted the creation of a 'function' for each of the temperature reaction setups (see Figure S4 and S5, Supporting Information) and, consequently, allowed us to calculate the *ee* of the ketone at any conversion. Finally, the effect of the temperature on enantioselectivity is exemplarily shown for conversions of 25%, 50%, and 75% in Figure 2, using the 'best shape' SLM function from Figure S5. SLM enabled us to clarify the trend of the 2,5-DKCMO system, by computing the *ee* of the ketone **1** for the same conversion, which was not obvious from the data collected since the different conversions obtained for the same reaction time obscured the trend (see Figure 2B). To give an idea, the *E* values calculated at 50% conversion from simulated *ee* of **1** ranged from 3 to 11 when the temperature decreased, as reported Table S1. Thus, we clearly demonstrated the effect occurred gradually as temperature changed.

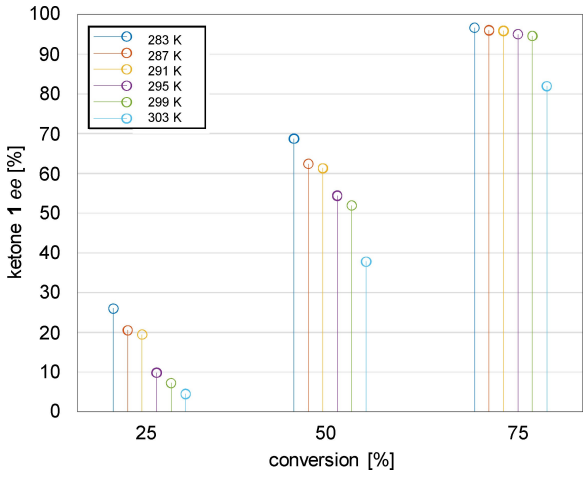


Figure 2. Enantiomeric excess (*ee*) of ketone **1** at conversions of 25%, 50%, and 75% from 283 K to 303 K simulated with the SLM approach.

The decreased stereospecificity of the system at higher temperatures could not be caused by a greater uncoupling of the flavin recycling system, which is (theoretically) possible in the presence of fully oxidized as well as reduced flavin and dioxygen in solution.[19] An uncoupling reaction would promote the chemical H_2O_2 oxidation of *rac*-**1** to the corresponding racemic normal lactone **2**, the abnormal lactone **3** being only formed enzymatically. However, the ratio of the 'abnormal' lactone enantiomer (+)-(1*S*,5*R*)-**3** over the normal lactone enantiomer (−)-(1*S*,5*R*)-**2** (both from (+)-(1*R*,5*S*)-**1**) was similar at the end of the reaction at all temperatures (3.0 ± 0.1 – data not shown) and thus confirmed that the observed temperature effect must be attributed exclusively to the monooxygenase-mediated BV oxidation of the model substrate.

The complexity of our reaction system hampers the usage of established methods, such as the determination of *E*. As the *E* value is an intrinsic property of an enzyme, it can only change if the intrinsic values of K_M or k_{cat} change,[16] and therefore must remain constant throughout the reaction, at any conversion. In our case, the classical calculation of *E* value revealed an increase with conversion (see Table S1). This observation is in line with Straathof and Jongejan comment, '*these values should be used with caution when conclusions are drawn about molecular effects on the intrinsic E value unless there is no doubt about the correctness of the assumptions on which the methods are based.*'[16]

As the enantiopreference of 2,5-DKCMO for (−)-(1*S*,5*R*)-**1** is decreasing with increasing temperatures (see Figure 2), there must be a (theoretical) 'racemic temperature' T_r, at which the enantioselectivity switches.[4b,7c,8c] Classically, T_r is determined by plotting $-RT\ln E$ *versus* *T*, in which T_r can be determined from the intercept of the curve with the abscissa.[4e] This methodology was not applicable to our system as the *E* values were inconsistence for different conversion. Nevertheless, additional experiments at temperatures until 313 K showed the enantioselectivity was maintained in favour of (−)-(1*S*,5*R*)-**1** conversion (maximum *ee* of **1**:10%, data not shown). We observed a considerable decrease of the enzymatic performance at the higher temperatures, which we address (at least) partially to enhanced enzyme degradation. Consequently, we expect the racemic temperature beyond the suitable operational temperature of the 2,5-DKCMO.

Although SLM enabled us to establish the gapless computation showing the entire enantioselectivity (for any conversion) of the system, the reaction mechanism of Type II BVMOs still remains uncertain. Most likely, it does not follow simple progress as it is required for the model for *E*.[17] Instead, a more complex reaction mechanism, e.g. sequential ordered,[20,21] may

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describe the system best (excluding the chemical reduction of the flavin cofactor as well as oxygen as a co-substrate, assuming it is in excess). On the other hand, in addition to the traditional rate constant representations, kinetics in catalysis may be analysed from an energy perspective. This view of energy terms in catalysis, e.g. the energetic spam model for catalytic cycles has recently gained more and more attention.[22] Consequently, energy-related terms should likewise be implemented in the kinetic concepts for enzymes. For example, the change in Gibbs free energy (ΔG^0) to reach the enzyme-transition state complex has been demonstrated to be important for the temperature dependence of enzyme kinetics.[23] However, even though the main concepts of catalysis can be transferred to biocatalysis, enzyme-catalysed reactions are often more complex. Consequently, further subtle effects, such as changes in the flexibility of the biocatalyst with temperature, or the heat capacity of the enzymes may be relevant.

Conclusion

We have demonstrated the temperature effect on the lactonization of bicyclo[3.2.0]hept-2-en-6-one **1**, a model substrate for BV oxidations, by 2,5-DKCMO, a rare representative of FMN-dependent BVMOs, with a greater enantiopreference at lower temperatures. The classical criterium to quantify stereospecificity, *E*, being not calculable with precision for this complex system, the 'Shape Language Modeling' (SLM) was introduced to reveal the temperature-dependent effect on the regio- and enantioselectivity of the biocatalyst unambiguously. Thus, we showed that SLM is an efficient tool to create prescriptive functions usefully applicable for reactions of unknown mechanistic details.

Experimental Section

Chemicals: All chemicals were utilized as supplied without further purification. Authentic lactone samples were synthesized by microbiological biotransformation according to described procedures.[24] AmNAH was synthesized as described in the literature.[25]

Enzyme preparation: The cloning, recombinant expression, and purification of the 2,5-DKCMO is described elsewhere.[11] The enzyme was stored −20 °C after purification without further processing and applied in the reactions from its frozen form.

Biotransformations: These were performed in closed 2 mL glass vials with a total volume of 1 mL in Tricine buffer (50 mM, pH 8.5) for final concentrations of 4 mM *rac*-ketone **1**, 25 mM AmNAH, 5.0 μM FMN, 0.19 mg mL^{-1} 2,5-DKCMO and 2.5 mg mL^{-1} catalase. The substrate was supplied from a 100 mM stock solution in ethanol (to a final ethanol content of 5% v/v). All reactions took place in an Innova® 42 Incubator Shaker (Eppendorf) at 160 rpm and various temperatures (283–303 K). Samples were taken from the reaction medium and extracted by a 0.5 g L^{-1} solution of tridecane (as internal standard) in ethyl acetate, in a volumetric ratio of 1-to-2. All experiments were performed in biologically independent duplicates.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: chemoenzymatic Baeyer-Villiger oxidation · kinetic resolution · Shape Language Modeling · temperature-dependent enantioselectivity

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