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Asymmetric Baeyer–Villiger Biooxidation of α-Substituted Cyanocyclohexanones: Influence of the Substituent Length on Regio- and Enantioselectivity

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Abstract: Cells of *Escherichia coli* expressing cyclohexanone monooxygenase catalyzed a highly enantioselective Baeyer–Villiger oxidation of α-substituted cyanocyclohexanones, leading to the corresponding enantiopure caprolactones (*ee*s >97%). Classical kinetic resolution and regiodivergent parallel kinetic resolution patterns were observed, depending on the length of the linear chain at the alpha position. Regioselectivity was compared with that of chemical BV oxidations. Cells revealed an outstanding stability since 50% of the specific BV activity was retained after 42 days of storage, which facilitates their use in biotransformations.

Keywords: asymmetric Baeyer–Villiger oxidation; biotransformation; cyano-lactone; cyclohexanone monooxygenase; regioselectivity; whole-cell process

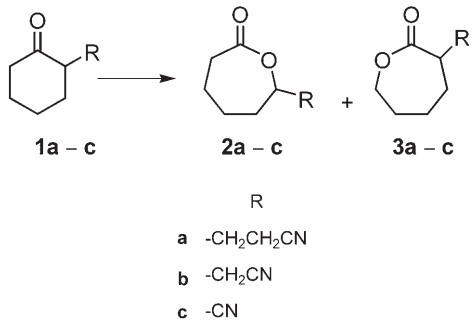
Introduction

Enzyme-mediated Baeyer–Villiger (BV) oxidation is nowadays largely recognized as an efficient method to obtain enantiopure lactones.[1] Starting from cyclic ketones, whole-cell biotransformations could be performed routinely at a preparative scale (several grams) on various substrates using an adsorbent resin-based process.[2] However, each new target compound implies first a screening of enzymes to find a highly enantioselective biotransformation (or even an enzyme redesign[3]) and subsequently a specific optimization step. The overall process is of course highly time-consuming. In another way, setting up some judiciously chosen biotransformations of versatile substrates could open an easier route to various enantiopure lactones. In this context, ketones bearing a cyano substituent are promising since the nitrile moiety is a functional group giving an easy access to amides, acids or amines and consequently to a great choice of other functionalities.

Given that, up to now, cyano-substituted ketones have never been studied as Baeyer–Villiger monooxygenase substrates (BVMOs), we evaluated the capacity of cyclohexanone monooxygenase (CHMO) to oxidize the α-substituted cyanocyclohexanones **1a–c** into the corresponding caprolactones in an enantioselective manner and with limited side-reactions (Scheme 1). We wish also to draw the attention to a simple experimental hint affording easier whole-cell biotransformations.

Results and Discussion

Because, up to now, whole cell-based BV oxidation generally turns out to be more convenient and appropriate for synthetic purposes than isolated enzyme-based process, a microbial biocatalyst was chosen. The overexpressing cyclohexanone monooxygenase



Scheme 1. Microbial Baeyer–Villiger oxidation of **1a–c** by overexpressing CHMO recombinant *E. coli*.

Table 1. Preparative-scale biotransformations of α-substituted cyanocyclohexanones.

R	Reaction time	Ketone **1**			Proximal lactone **2**			Distal lactone **3**		
		Yield[a]	*ee*[b]	Absolute conf. $[\alpha]_D^{30}$	Yield[a]	*ee*[b]	Absolute conf. $[\alpha]_D^{30}$	Yield[a]	*ee*[b]	Absolute conf. $[\alpha]_D^{30}$
$(CH_2)_2CN$, **a**	1 h 45 min	46 %	95 %	(*S*),[c] +14 (*c* 1, MeOH)	50 %	97 %	(*R*),[d] −100 (*c* 1.1 $CHCl_3$)	0	–	–
CH_2CN, **b**	2 h 15 min	0	–	–	32 %	>99 %	(*R*),[e] −23 (*c* 1 $CHCl_3$)	36 %	>99 %	(*S*), −30 (*c* 1, $CHCl_3$)

[a] Yields were based on isolated products.
[b] Ketone and lactone ees were determined by chiral GC (Chirasil Dex and Lipodex E columns) analysis.
[c] The absolute configuration of **1a** was assigned by comparison with the sign of the optical rotation given in ref.[7]
[d] The absolute configuration of **2a** was deduced from that of **1a**.
[e] The absolute configuration of **2b** was tentatively assigned by analogy with the optical rotation sign and the enantiomer elution order of **2a** on two chiral GC columns.

(CHMO) recombinant *E. coli* TOP10[pQR239][4] is endowed with an endogenous NADPH recycling system efficient enough to ensure preparative biotransformation in high substrate concentration as shown by our recent successful kilogram-scale experiments.[5]

Biotransformations of **1a** and **1b** were particularly fast since they reached completion in about two hours (*cf.* Table 1). Substrate **1c** did not undergo reaction even after 24 h.

The biotransformation of **1a** was entirely concordant with the classical scheme of a highly enantioselective kinetic resolution, displaying a high enantiomeric ratio (E > 200). The lactone was the proximal[6] regioisomer **2a**, rising from oxygen insertion between the carbonyl group and the substituted carbon atom. The absolute configurations were, respectively, (*S*) for the remaining ketone **1a** and (*R*) for the proximal caprolactone **2a**, in agreement with the enantioselectivity of CHMO generally observed with cycloalkanones substituted at the α position.[1]

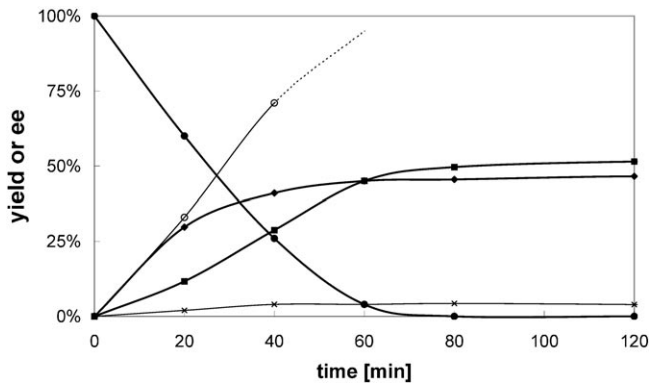


Figure 1. Time-course of biotransformation of ketone **1b** by 10 day old cells. ● residual ketone yield, ○ residual ketone *ee*, ◆ proximal lactone yield, ■ distal lactone yield, × alcohol yield.

By contrast, the biotransformation of **1b** corresponded to an almost perfect regiodivergent parallel kinetic resolution.[8] Both regioisomeric lactones, proximal **2b** and distal **3b**, were formed nearly simultaneously, each of them arising from a different ketone enantiomer. The formation of the proximal lactone was slightly faster than the distal one (Figure 1) and was strictly enantioselective as confirmed by the high lactone *ee*s (>99 %).

This type of reaction is not rare with BVMOs. It was already observed with bicyclic ketones,[9,10] 3-substituted[11] and 2,3-disubstituted cyclohexanones.[12] In the case of CHMO-mediated biotransformation on substrates displaying a low difference of electronegativity between potentially migrating carbon atoms, the regioselectivity reflected generally that observed with the chemical BV reagent mCPBA, as exemplified by 3-substituted cyclohexanones or the norbornanone family. On the contrary, in the case of fused bicyclobutanones[9] and dihydrocarvone,[12] a divergence was observed from the chemical reaction where a single lactone was predominantly formed.

In order to compare enzymatic and chemical BV oxidations on cyanocyclohexanones, we carried out classical oxidations with mCPBA and Na_2HPO_4. Ketone **1a** disappeared completely in 5 h. 30 % of ketone **1b** were still present after 72 h whereas no reaction was observed with ketone **1c**, even after 5 days. The regioselectivity of the reaction of **1a** was high, proximal lactone **2a** was almost exclusively formed. In the case of the cyanomethyl substituent (ketone **1b**), both regioisomeric lactones **2b** and **3b** were obtained in nearly 1:2 proportions (*cf.* Table 2).

These results, reaction rate and regioselectivity, reveal the influence of the electron-withdrawing nitrile group on the Criegee adduct rearrangement. As predicted by the well known rules governing BV oxidations,[13] the migration of the more electronegative carbon is favoured. The influence of the nitrile group

Table 2. Regioselectivity of chemical and biological Baeyer–Villiger oxidations.

	R	Ratio proximal lactone **2**:distal lactone **3**			
		mCPBA/Na_2HPO_4	mCPBA/TFA	30% H_2O_2/TFA	CHMO
a	$(CH_2)_2CN$	15:1	6:1	4:1	1:0
b	CH_2CN	1:1.7	1:10	1:12	1:1
c	CN	nr	nr	nr	nr

nr: no reaction.

of the cyanoethyl substituent (ketone **1a**) did not suffice to orient the migration. As usually observed, the migrating group was the most substituted one. On the other hand, the carbon atom bearing the cyanomethyl substituent was less electronegative than the unsubstituted carbon atom on the other side of the carbonyl group. Both migrated but the reaction was considerably slowed down in comparison with the cyanoethyl substituent. Interestingly, when trifluoroacetic acid (TFA), usually used as an acid catalyst to accelerate BV oxidation, is employed with mCPBA,[14] reactions were faster (19 h instead of 72 h for **1b**) and distal lactone formation increased in such extent that **3b** was formed predominantly (*cf.* Table 2). The use of trifluoroperacetic acid, a more powerful reagent,[13] accentuated this trend.[15]

To summarize, CHMO-mediated biotransformations of methylcyano- and ethylcyanocyclohexanones displayed a regioselectivity close to that of basic catalyzed chemical BV oxidation that underlines the importance of the intrinsic reactivity of Criegee intermediates. Up to now, regio- and enantioselectivity were explained by the impossibility, essentially due to steric hindrance, for a ketone enantiomer to be accommodated into the active site and/or for the Criegee intermediate to adopt the antiperiplanar configuration[16] required for its rearrangement. Thus, the compilation of results concerning CHMO-mediated BV oxidation led to several empirical models (cubic[9,12,17], diamond lattice[11]) that define various "forbidden" or "allowed" areas. However, according to that explanation, the result of the biotransormation of **1b** is still quite unexpected when compared to biotransformations of the sterically close 2-alkylated cyclohexanones with the same enzyme. Only a single enantiopure proximal caprolactone was observed whatever the length of the alkyl chain,[18] that means only one ketone enantiomer was oxidized. On the other hand, the energy of the Criegee intermediate conformation corresponding to the distal lactone formation does not seem so high that the rearrangement was impossible.[19] So, in that peculiar case of **1b**, we suggest a specific binding interaction of the cyano group into the active site which would exist neither with apolar substituents similar in steric hindrance, like ethyl or propyl, nor with the ethylcyano substituent.[20] Such an interaction could also influence conformational equilibria which have probably to be considered as well as the reactivity of Criegee intermediates. Nevertheless, it will be difficult to reach a better and more general understanding of the observed enantioselection as long as the 3D structure of CHMO is not known.

The direct cyano substitution (ketone **1c**), instead favouring the non-substituted carbon towards migration as expected, seemed to prevent the occurrence of BV oxidation whatever the reagent, enzyme or peracids. That could result from the conjugation between carbonyl and cyano group which prevents the nucleophilic attack at the carbonyl group.

The disadvantage of the use of compounds bearing cyano groups is that they can undergo unwanted side-reactions, particularly when whole cells are used since active nitrilases and nitrile hydratases are present in numerous strains. In our case, high yields in cyanolactones suggested the absence of these activities, which was corroborated by the fact that none of these enzymes were found in *E. coli* strains.[21] As frequently observed, alcohols were sometimes formed in low yield (<4%).

Experimental Hint

The high level of enantioselectivity and obtained yields prompted us to continue our work aimed at the improvement, from a practical standpoint, of whole cell-mediated biooxidations with recombinant *E. coli* TOP10[pQR239].[22] Biotransformations were generally carried out directly in culture medium immediately after growth or, at the latest, the day after. In these conditions, the overall duration of one experiment cannot be less than two days in the best cases and required tedious handling. To be closer to the convenience of a ready-on-shelf reagent, we investigated how to keep the cells active as long as possible. It appeared that cells whose growth was stopped before the stationary phase and one or two hours after induction, were still active 61 days later provided that they were conserved at 4°C (*cf.* Figure 2). Cell broth was diluted with one tenth or one fifth of fresh medium then placed into a flask or a fermentor in

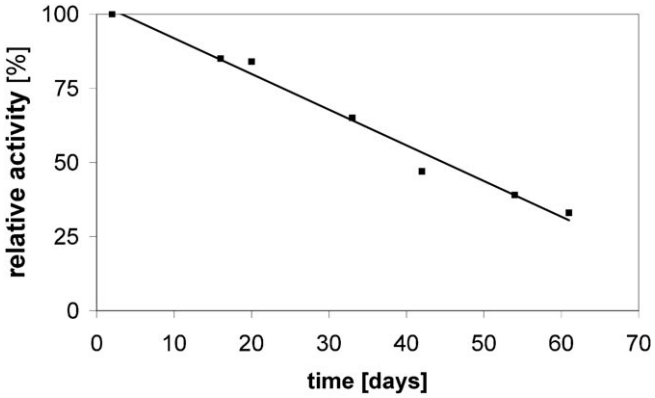


Figure 2. Relative activity of cells against bicyclo[3.2.0]hept-2-en-6-one *versus* 4 °C storage time.

biotransformation conditions for 30 to 60 min. The biotransformation was conducted as usual although the cell concentration was relatively low ($OD_{590nm} = 5$ instead of 12). As shown by Woodley's group,[23] this parameter is not a limiting factor for an efficient biotransformation. The effect of high concentrations can even be adverse if cells use the totality of the available oxygen for metabolism and not for BV oxidation. Thus, depending on the reactor's capacity to supply oxygen, it can be worthwhile working at a lower level of cell concentration. In our case, the specific cell activity after 42 days was almost 50 % of the initial value, which still allows a good level of biotransformations. To the best of our knowledge, such a high stability of recombinant cell activity has never been described and offers interesting opening towards efficient whole-cell process developments.

Conclusions

The three nearly enantiopure caprolactones **2a**, **2b** and **3b** were synthesized in high yields by microbial BV oxidation of substituted cyclohexanones bearing a cyano group at the end of a linear chain. Considering the essential role of the nitrile function in generation of various other functional groups, these biotransformations are good candidates for a future large scale optimization more especially as the surprisingly high stability of whole cells of *E. coli* TOP10[pQR239] affords an undeniable practical advantage.

Experimental Section

The recombinant *E. coli* TOP10[pQR239] overexpressing cyclohexanone monooxygenase (EC 1.14.13.22) was constructed by Ward.[4]

2-Cyanoethylcyclohexanone **1a** was commercially available (Acros). 2-Cyanomethylcyclohexanone **1b** (prepared *via* Stork alkylation,[24] 55 % yield) and 2-cyanocyclohexanone **1c** (Thorpe–Ziegler condensation of pimelonitrile,[25] 70 % yield) were obtained according to the literature procedures.

All fermentation media components [soybean peptone (70178), yeast extract (70161), glycerol, ampicillin, L-(+)-arabinose] were kindly supplied by Fluka.

A quick investigation of the growth medium composition revealed the importance of the peptone origin. Better growth was observed with peptones from soybean ($OD_{590nm} = 12–18$) than with peptones from casein or bacteriological peptones ($OD_{590nm} = 5–10$) while specific activity (correlated to cell dry weight) with a peptone from soybean meal was 5-fold higher than peptones from casein.

Cell cultures were conducted in a 10-L fermentor as previously described.[26] When OD_{590nm} reached about 4, arabinose (0.05 % w/v) was added and growth was continued for one hour. After stopping, the cell broth was placed at 4 °C in a non-sealed bottle and taken when needed. Cell broth (800 mL), fresh culture medium (200 mL) and ampicillin (100 mg) were introduced into a 2-L fermentor (30 °C, 550 rpm, 0.17 vvm air). pH was maintained at 7.1 by addition of KOH solution. DOT (dissolved oxygen tension) quickly reached a value close to zero, indicating a resumption of the growth. After 30–45 min, ketone (1 g in 10 mL EtOH) was added. Biotransformation was monitored by GC analysis (Optima 5 amino column – Macherey–Nagel) of samples taken at regular intervals until completion. Broth pH was set at 5 before continuous extraction with CH_2Cl_2 (4×8 h). Flash chromatography (pentane/AcOEt) then bulb-to-bulb distillation led to purified products. The *ee*s of ketones (respectively lactones) were determined on Macherey–Nagel Lipodex®E column (respectively Chrompack Chirasil-Dex® CB column).

Cell stability was determined with two substrates: bicyclo-[3.2.0]hept-2-pn-6-one, classically used to calculate cell specific activity, and ketone **1a**. Biotransformations were carried out in a 250-mL flask at 30 °C. Bicyclo[3.2.0]heptenone (10 mg in 100 μL of EtOH) was added to cell broth (9 mL), 0.5 M phosphate buffer solution (9 mL), and fresh culture medium (2 mL). Cell specific activity calculations were based on substrate disappearance determined by GC analysis. Ketone **1a** (20 mg) was transformed by cell broth (18 mL) diluted with 2 mL of fresh medium after pH adjustment at 7.2 if needed.

Supporting Information

Spectroscopic data of lactones **2b** and **3b**, time-course of **1a** biotransformation by 33- and 44-day old cells are given in the Supporting Information.

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