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Developing a Biocascade Process: Concurrent Ketone Reduction-Nitrile Hydrolysis of 2-Oxocycloalkanecarbonitriles

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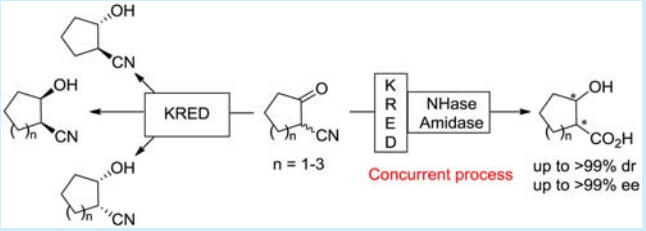
Supporting Information

ABSTRACT: A stereoselective bioreduction of 2-oxocycloalkanecarbonitriles was concurrently coupled to a whole cell-catalyzed nitrile hydrolysis in one-pot. The first step, mediated by ketoreductases, involved a dynamic reductive kinetic resolution, which led to 2-hydroxycycloalkanenitriles in very high enantio- and diastereomeric ratios. Then, the simultaneous exposure to nitrile hydratase and amidase from whole cells of *Rhodococcus rhodochrous* provided the corresponding 2-hydroxycycloalkanecarboxylic acids with excellent overall yield and optical purity for the all-enzymatic cascade.

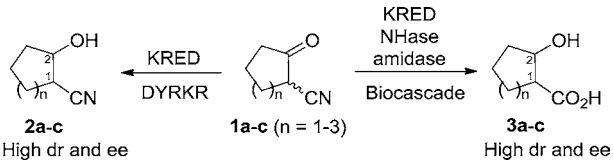


Oxidative−reductive transformations belong to the most important reactions in organic synthesis. Redox-active enzymes such as oxygenases, alcohol dehydrogenases, amine dehydrogenases, and ene-reductases selectively catalyze the introduction and modification of functional groups under mild reaction conditions and can create chiral centers with excellent stereoselectivity. Although some critical issues such as cofactor-dependency, regeneration systems, or unfavorable reaction equilibria are associated with these processes, different approaches such as the use of biomimetic cofactors[1] or the coupling of several enzymatic steps have been developed.[2]

However, the possibility of performing multistep enzymatic synthesis in a concurrent fashion is particularly appealing since this strategy can reduce costs significantly. This fact, combined to the intrinsic green features of enzymes, increase the sustainability expectations of biocatalysis-based chemical manufacturing. Multistep reactions in whole cell fashion were described as early as in the 1980s for the production of amino acids.[3] As for the possibilities to combine several isolated enzyme classes facilitating a one-pot cascade, the use of redox enzymes has been successfully demonstrated in recent years.[2]

Early reports on the bioreduction of cyclic β-ketonitriles relied on biotransformations by fungi and yeasts, leading to the corresponding *cis*-(1*S*,2*S*)-β-hydroxy nitriles in high enantio- and diastereomeric excess.[4] More recently, isolated carbonyl reductases were sequentially coupled with nitrilases to provide enantiopure linear β-hydroxy carboxylic acids from α-unsubstituted β-ketonitriles.[5] In this sense, an interesting alternative to nitrilases could be the use of two enzymes, nitrile-hydratase and amidase, which are found in different strains of *Rhodococci*. In the light of this background, we envisaged to test the bioreduction of several 2-oxocycloalkanecarbonitriles (**1a−c**) concurrent to the enzymatic hydrolysis of the cyano group (Scheme 1). Thus,

Scheme 1. KRED and Enzymatic Cascade for Transforming 2-Oxocycloalkanecarbonitriles



taking advantage of the epimerizable stereocenter of **1a−c** (pK_a = 7.84 for **1a**),[4] our first goal was to identify highly stereoselective ketoreductases (KREDs) to promote efficient dynamic reductive kinetic resolutions (DYRKR).[6] Then, the resulting optically active β-hydroxynitriles **2a−c** would undergo, by the successive action of a nitrile-hydratase (NHase) and an amidase, a further hydrolysis to the final β-hydroxyacids **3a−c**. However, although water is the natural enzyme environment, a number of issues should be addressed to implement such enzymatic cascade.[7] A major challenge is the compatibility of the different enzymes with the preferred pH, temperature, concentration, and cosolvents, but also specific activities and stability should be balanced and inhibition avoided. In addition, to set a process as shown in Scheme 1, the tandem NHase-amidase system should ideally not be active toward the starting β-ketonitrile, or in the worst case, much slower than toward the intermediate β-hydroxynitrile.

The starting β-ketonitriles **1a−c** were synthesized from inexpensive alkanedinitriles following literature procedures.[4] Then, the bioreduction was tested using ketoreductases from the Codex KRED Screening Kit with isopropanol (IPA) for cofactor recycling and as cosolvent. Initially, the screening was performed

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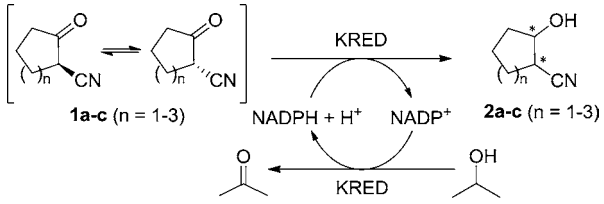
under the standard conditions at pH 7.0 (Tables S1, S3, and S5),[8] which should be enough to promote the desired racemization. Alternatively, bioreductions of **1a−c** at pH 5.0 and 10 were also tested (Tables S2 and S4).[8] Thus, all the KREDs led to complete conversion after 24 h, and the resulting 2-hydroxycycloalkanenitriles were isolated in high yield (>95%). With regards to the stereoselectivity, results were biocatalyst- and ketone-dependent, the most representative are shown in Table 1.

Table 1. DYRKR of β-Ketonitriles 1a−c Catalyzed by KREDs[a,b]



entry	β-ketonitrile	KRED	*cis*/*trans*	ee_{cis} (%)[e,f]	ee_{trans} (%)[e,f]
1[c]	**1a**	P2-D11	10:90		>99 (1*R*,2*S*)
2[d]	**1a**	P2-D11	6:94		>99 (1*R*,2*S*)
3[c]	**1a**	P1-B12	83:17	98 (1*R*,2*R*)	
4[d]	**1a**	P1-B12	88:12	>99 (1*R*,2*R*)	
5[c]	**1a**	P2-G03	96:4	90 (1*S*,2*S*)	
6[d]	**1a**	P2-G03	98:2	95 (1*S*,2*S*)	
7[d]	**1a**	NADH101	>99:<1	>99 (1*S*,2*S*)	
8[c]	**1b**	P1-A04	>99:<1	>99 (1*S*,2*S*)	
9[c]	**1b**	P2-H07	>99:<1	>99 (1*S*,2*S*)	
10[c]	**1b**	P1-B10	94:6	>99 (1*R*,2*R*)	
11[c]	**1b**	P1-B12	>99:<1	>99 (1*R*,2*R*)	
12[c]	**1c**	P1- A04	>99:<1	>99 (1*S*,2*S*)	
13[c]	**1c**	P1- B10	98:2	>99 (1*R*,2*R*)	

[a]Substrate (20 mM) in KH_2PO_4 buffer, 125 mM (1.25 mM $MgSO_4$, 1 mM $NADP^+$), pH 7.0 or 5.0 (900 μL), KRED (2 mg), IPA (190 μL), 24 h at 250 rpm and 30 °C. [b]Conversion >99%. [c]pH = 7.0. [d]pH = 5.0. [e]Measured by chiral GC. [f]Absolute configuration established as detailed in the SI.

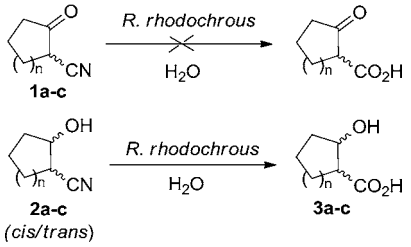
In the case of the cyclopentanone derivative **1a** (entries 1−7), half of KREDs exhibited *cis* diastereoselectivity; meanwhile, the other half afforded predominantly the *trans*-counterpart, the best results being obtained at pH 5.0. Pleasantly, we could identify biocatalysts that gave rise to three out of the four possible stereoisomers with high diastereomeric ratio and enantiomeric excess >99%. Remarkably, KRED-P2-D11 yielded the *trans* isomer (1*R*,2*S*)-**2a** [(1*R*,2*S*)-2-hydroxycyclopentanecarbonitrile] in 16:1 dr and >99% ee (entry 2). Regarding the *cis*-isomer, KRED-P1-B12 provided enantiopure (1*R*,2*R*)-**2a** with 7:1 dr (entry 4); meanwhile, KRED-NADH101 gave its counterpart (1*S*,2*S*)-**2a** with an excellent dr and >99% ee (entry 7).

In the case of the cyclohexanone derivative **1b**, all the KREDs afforded the *cis*-diastereomer predominantly, with better performances at pH 7.0. It is noteworthy the case of KRED-P1-A04 and KRED-P2-H07, which exhibited total selectivity toward (1*S*,2*S*)-**2b**, with >99 dr and >99% ee (entries 8−9). Likewise, the enantiomer (1*R*,2*R*)-**2b** could also be obtained in 32:1 dr and >99% ee from the reaction catalyzed by KRED-P1-B12 (entry 11). The best KREDs found in the bioreduction of **1b** remained the same for its seven-membered analogous **1c** (entries 12−13), again favoring the formation of the *cis*-diastereomer. Thus, KRED-P1-A04 rendered, by means of a highly efficacious DYRKR, (1*S*,2*S*)-**2c** in >99 dr and >99% ee. It is worth mentioning that both enantiomers of *cis*-2-hydroxycycloheptanenitrile were prepared in this report for the first time.

Nitriles are immediate precursors of carboxylic acids, but harsh conditions typically used for their hydrolysis are often incompatible with most other functional groups.[9] The biotransformation of nitriles, however, either through a direct conversion to a carboxylic acid catalyzed by a nitrilase or through the previous NHase-catalyzed hydration followed by the amidase-catalyzed hydrolysis of the resulting amide, is a conveniently mild alternative.[10] In this regard, several *Rhodococci* catalyzed the hydrolysis of closely related cyclic *N*-protected-β-aminonitriles with comparable trends: five-membered substrates were transformed significantly faster than the six-membered homologues, and the *trans*-derivatives reacted faster than the *cis*-counterparts. Moreover, the enantioselectivity was higher for the *trans*-isomers.[11]

Initial screening experiments were performed with both β-ketonitrile **1a** and a *cis*/*trans* mixture of β-hydroxynitrile **2a** employing nitrilases from the Codex Nitrilase Screening Kit, but all the attempts showed low activities. Accordingly, we focused our attention on whole-cell biocatalysts, namely, the commercially available bacterium *Rhodococcus rhodochrous* IFO 15564.[12] Thus, biotransformations of **1a−c** and **2a−c** were performed with a standard cell concentration of microorganism in the metabolic resting phase [approximately 0.9 mg/mL of aqueous 0.1 M phosphate buffer pH 8.0, 1% EtOH v/v (equivalent to A_{650} = 1.0)]. TLC analysis after 24 h showed complete conversion of *cis*- and *trans*-isomers of **2a−c** into the corresponding β-hydroxyacids **3a−c** and no reaction with the β-ketonitriles **1a−c** (Scheme 2). Interestingly, both facts fulfilled the prerequisites

Scheme 2. Preliminary Study of *R. rhodochrous* Activity



for the implementation of the biocascade: (1) the starting material is substrate only for the KRED; (2) the tandem NHase-amidase is very active toward the product of the KRED, independently of its absolute configuration. Accordingly, the selectivity of the biocatalyst was not analyzed in depth since the second step of the cascade will be fueled with optically active β-hydroxynitriles in very high dr and ee. Even so, *R. rhodochrous* displayed higher reactivity and enantioselectivity toward the *trans*-isomers (data not shown), in accordance with the previous background.[11]

Next, we took the challenge of coupling both steps into a concurrent process with both enzymes working "hand-in hand". Actually, the previous report dealing with linear β-ketonitriles was performed sequentially, and, once the carbonyl reductase-catalyzed reaction was completed, the pH was readjusted and the nitrilase added to the medium.[5] In preliminary cascade attempts, **1b** was subjected to the medium used in the bioreductions but containing both *R. rhodochrous* IFO-15564 and a KRED. Regarding the cosolvent, IPA (essential for KREDs) and EtOH

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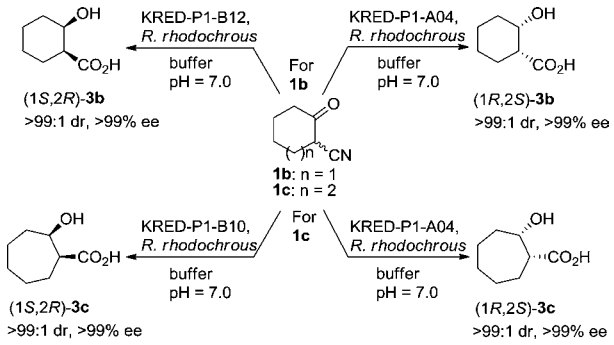
(typical with *R. rhodochrous*) were checked in different ratios (1−15% v/v). The most significant outcomes were the following: (1) EtOH is not accepted for KREDs; (2) 15% v/v of IPA inhibits the activity of *R. rhodochrous* at A_{650} = 1−4; (3) both *R. rhodochrous* (A_{650} = 3−4) and KRED are active with 5% v/v of IPA; and (4) the activity of *R. rhodochrous* significantly decreased at pH 5. Based on this parametrization, the biotransformation of β-ketonitriles were designed using phosphate buffer (125 mM, 1.25 mM $MgSO_4$, 1 mM $NADP^+$) pH 7.0, IPA 5% v/v, and whole cells of A_{650} = 4. Under these optimized conditions, both biocatalysts were functional and led to the target β-hydroxyacids.

All the reactions assayed at pH 7.0 in Table 1 were submitted to the biocascade. Thus, both cyclohexylic and cycloheptylic β-ketonitriles **1b** and **1c** underwent effective bioconversion into the corresponding β-hydroxyacids **3b** and **3c** with nearly the same dr and ee (selected examples in Scheme 3).[13] Regarding **1b**, the

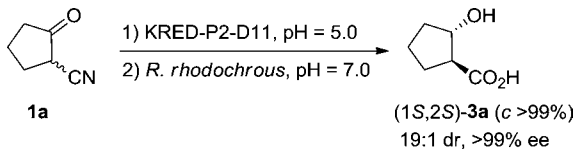
Scheme 3. Biocascade Towards Stereoisomers of 3b and 3c



combination of *R. rhodochrous* IFO-15564 and the stereocomplementary ketoreductases P1-A04 and P1-B12 delivered, respectively, (1*R*,2*S*)-**3b** and (1*S*,2*R*)-**3b** in >95% yield (30 mg scale) and very high selectivity: >99:1 dr and >99% ee (Scheme 3). Further extension to **1c** provided both antipodes of the previously unreported *cis*-2-hydroxycycloheptanecarboxylic acid (**3c**), namely, (1*R*,2*S*)-**3c** and (1*S*,2*R*)-**3c** in nearly quantitative yield and complete selectivity (>99 dr and >99% ee).

Although operationally viable, the cascade for the 5-membered ring homologue **1a** was challenging since the optimal selectivity in the bioreduction step was achieved at pH 5.0 (Table 1, entries 1−7), which inhibits the activity of *R. rhodochrous*. Accordingly, we turned our attention toward a stepwise process. Thus, a 30 mg-scale bioreduction of **1a** was performed with KRED-P2-D11 at pH 5.0, and once the reduction was completed, pH was raised to 7.0 and a bacterial suspension of high absorbance (A_{650} = 4) added. As a result, (1*S*,2*S*)-**3a** was isolated in very high dr (19:1) and excellent ee (>99%), despite a moderate yield of 52% due to the incomplete extraction from the aqueous medium (Scheme 4).[14] It is of note the simplicity of the setup, despite not being a "true cascade" since the isolation of the β-hydroxynitrile intermediate was not necessary. Actually, both steps were performed under identical reaction medium, and a change on the

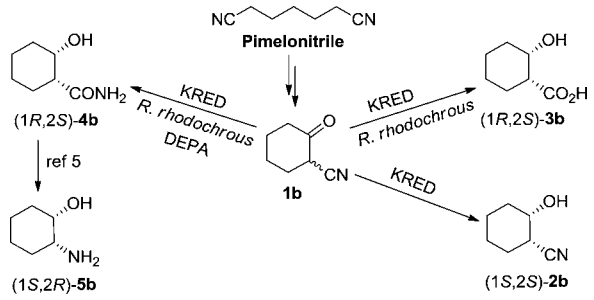
Scheme 4. One-Pot Sequential Synthesis of (1*S*,2*S*)-3a



pH was the only adjustment before the addition of the microorganism.

Finally, to exploit the synthetic utility of the enzymatic platform developed herein, a biocascade using **1b** was carried out with whole cells of *R. rhodochrous* grown in the presence of DEPA, an inhibitor of the amidase activity of the microorganism (Scheme 5). Under these conditions, (1*R*,2*S*)-2-hydroxycyclo-

Scheme 5. Enzymatic Platform Towards Valuable Optically Active Compounds Starting from Alkanedinitriles



hexanecarboxamide (**4b**) was isolated from the cascade with KRED-P1-A04. This compound is also an immediate precursor of (1*S*,2*R*)-*cis*-2-aminocyclohexanol (**5b**), completing a spectrum of valuable optically active molecules from inexpensive pimelonitrile, as exemplified in Scheme 5.

In summary, we have developed an enzymatic cascade process in aqueous medium combining a highly selective DYRKR of 2-oxocycloalkanenitriles, mediated by KREDs, with whole cells containing NHase-amidase activity for a further nitrile hydrolysis. Thus, the success of the strategy lied both in the synchronization between a fast racemization compared to the bioreduction of the nonpreferred enantiomer in the first step, and a microorganism that converts the cyano group of the β-hydroxynitrile intermediate but not the one contained in the starting β-ketoxynitrile. Interestingly, the KREDs showed markedly *cis*-diastereoselectivity, complementing the existing methodologies, mostly aimed at *trans*-diastereoisomers, readily available from epoxides and aziridines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b01510.

Experimental procedures, enzymatic screenings, characterization data, copies of the corresponding ^1H, ^{13}C spectra, and GC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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(8) See Supporting Information for all the enzymatic screenings.

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(13) For **3a-c**, the Cahn−Ingold−Prelog priority of the substituents at the C-1 position changed with respect to **2a-c**.

(14) The starting material **1a** and the intermediate **2a** were not detected, which indicates that the sequential process worked efficiently.

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