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Biocatalysis from the perspective of an industrial practitioner: let a biocatalyst do a job that no chemocatalyst can

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Dedicated to Professor Takeshi Kitahara on the occasion of his 61st birthday and retirement from the University of Tokyo

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Abstract

For biocatalysis-based processes to be scalable, one has to fulfill at least three requirements: (1) industrially acceptable throughput (volume efficiency); (2) prevention of product inhibition; (3) product separation by partition without recourse to chromatography; and in the case of kinetic resolution, the off-enantiomer needs to be recycled. In the chemoenzymatic synthesis of (*R*)-3-*tert*-butoxycarbonyl-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (BocDMTA) **1** featuring a *Klebsiella oxytoca* hydrolase, the thermal stability of the enzyme helps to attain an industrially feasible concentration of the substrate, methyl (±)-5,5-dimethyl-1,3-thiazoline-4-carboxylate **6b**, at 60 °C: [(±)-**6b**] = 3.0 M (575 g/L). In the (*S*)-selective hydrolysis of (±)-3-butyryloxyquinuclidinium butyrate **12** with an *Aspergillus melleus* protease, $Ca(OH)_2$ serves as so effective a scavenger of butyric acid as to prevent it from impeding the catalytic activity of the protease. This allows the enzymatic hydrolysis to proceed at [(±)-**12**] = 2.0 M (571 g/L); on extractive separation from the left-over (*R*)-3-quinuclidinyl butyrate **11a**, which is converted to (*R*)-3-quinuclidinol **2** via methanolysis, the digested (*S*)-**2** can be racemized over Raney Co under hydrogen for another round of the enzymatic resolution. In the synthesis of *trans*-1-(1,3-dihydroxypropan-2-yl)-4-propylcyclohexane **3**, *cis*-4-propylcyclohexanol **14** is prepared by *Galactomyces geotrichum*-mediated equatorial hydride delivery to 4-propylcyclohexanone **15**; while the microbial reduction fails to go to completion, the unconsumed ketone **15** can be removed via bisulfite adduct formation or by simple distillation after malonate homologation.

Keywords: Biocatalysis; Enantioselective hydrolysis; Equatorial hydride delivery

1. Introduction

Back in the 1980s when molecular catalysis was still in its infancy, microbial catalysis helped synthetic chemists build stereogenic centers more than now [1–3]. However, things have changed dramatically over the past two decades as synthetic chemists have succeeded in competing with hydrolytic enzymes in non-aqueous media [4–6] to say nothing of Noyori's triumph over baker's yeast [7–9].

Will biocatalysis bow out in the new millennium then? The purpose of this article is to disprove such negative notion against biocatalysis by discussing three case studies on its application from an industrial viewpoint [10]: (1) a *Klebsiella oxytoca* hydrolase-mediated kinetic resolution of chiral, racemic carboxylic acid in a practical assemblage of (*R*)-3-*tert*-butoxycarbonyl-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (BocDMTA) **1** (Fig. 1) [11]; (2) an *Aspergillus melleus* protease-mediated kinetic resolution of chiral, racemic alcohol in a scalable preparation of (*R*)-3-quinuclidinol **2** (Fig. 2) [12]; (3) *Galactomyces geotrichum*-mediated *cis*-selective reduction of 4-propylcyclohexanone [13] in a scalable synthesis of *trans*-1-(1,3-dihydroxypropan-2-yl)-4-propylcyclohexane **3** (Scheme 6).

To adapt biocatalysis, whether it employs isolated enzymes or whole cells, for industrial production, its throughput needs to be increased to an industrially viable level and hence substrate concentration should be maximized while biocatalyst loadings are kept to a minimum. Under those constrained conditions, biocatalysis is sometimes plagued with product inhibition, a phenomenon that is often overlooked in the laboratory where heavy loads of biocatalysts

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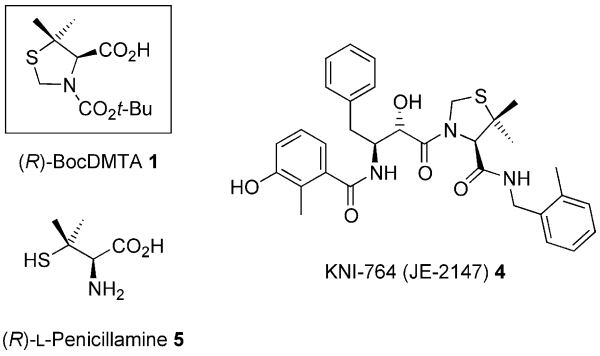


Fig. 1. Structures of (*R*)-BocDMTA **1**, KNI-764 **4**, and (*R*)-L-penicillamine **5** [15].

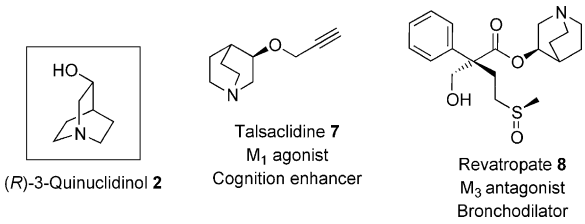


Fig. 2. Structures of (*R*)-3-quinuclidinol **2**, talsaclidine **7** [23] and revatropate **8** [24].

can be used under dilute conditions [14]. Moreover, even if such product inhibition could be overcome somehow or other, biocatalysis would remain difficult to scale up without a practical method to isolate a desired product [14]. Lastly, when biocatalysis is applied to kinetic resolution, an off-enantiomer should be recovered and then racemized to gain >50% of atom efficiency with respect to the racemic substrate [14].

Thus, in the three case studies that follow, foci will be placed on tactics that have been designed: (1) to maximize throughput (volume efficiency); (2) to rescue biocatalysis from product inhibition; (3) to enable practical product separation; (4) to racemize an off-enantiomer for its reuse in the case of kinetic resolution.

2. Chemoenzymatic preparation of (*R*)-BocDMTA 1: a thermally stable hydrolase helps attain industrially viable throughput

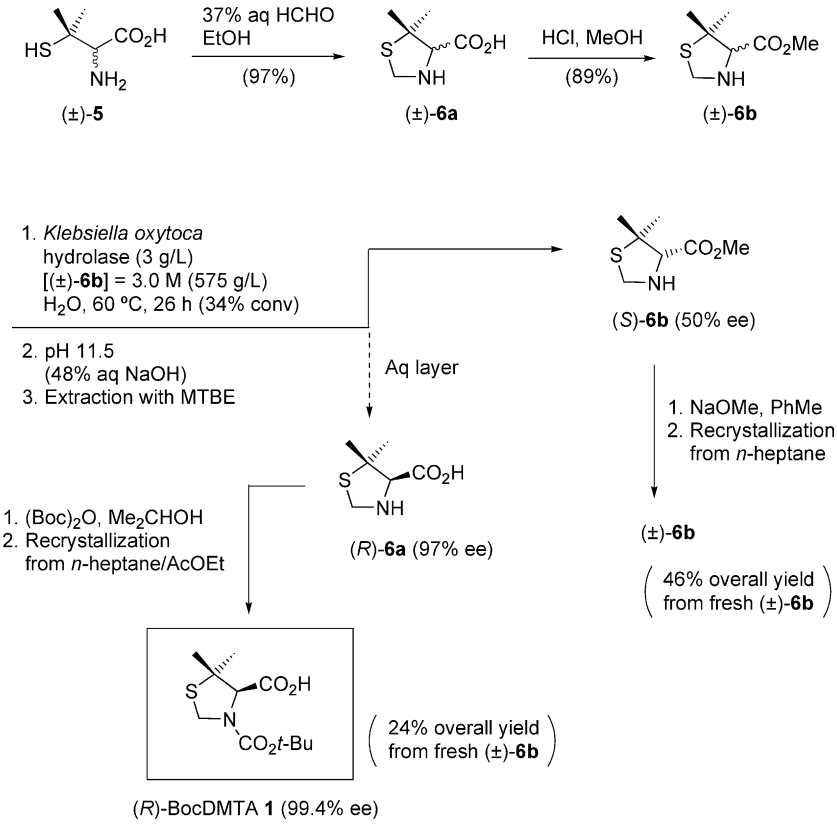
(*R*)-BocDMTA **1**, a key building block for a potent HIV protease inhibitor, KNI-764 **4**, might be prepared from (*R*)-L-penicillamine **5** without difficulty once (*R*)-BocDMTA **1** could be recognized as a plain derivative of (*R*)-**5** (Fig. 1) [15]. However, in contrast to natural (*S*)-D-penicillamine **5** that is prescribed as an antidote to heavy metals, unnatural less available (*R*)-L-penicillamine **5** is notorious for causing optic atrophy that sometimes leads to blindness [16]. Therefore, to have a practical access to (*R*)-BocDMTA **1**, another method should be developed that could dispense with (*R*)-L-penicillamine **5**, and as such, kinetic resolution of methyl (±)-5,5-dimethyl-1,3-thiazolidine-4-carboxylate **6b** by enzymatic enantioselective hydrolysis was explored (Scheme 1) [11].

Commercially available (±)-penicillamine **5** was converted into racemic methyl ester **6b** according to known procedures consisting of *N*,*S*-acetal formation followed by acid-catalyzed esterification (Scheme 1) [11,17]. Forty-three different hydrolase preparations (lipases, proteases and esterases) were tested for the (*R*)-selective hydrolysis of (±)-**6b** and a *Klebsiella oxytoca* hydrolase was identified as the chiral discriminator of choice: when (±)-**6b** (90 mM) was treated with the hydrolase {0.1% (w/v)} in an unbuffered aqueous medium at 25 °C for 24 h, (*R*)-**6a** was produced in 99.7% ee with $E > 500$ [18] at 41.6% conversion as monitored by chiral HPLC [Sumichiral OA-5000 (Sumika); 2 mM solution of $CuSO_4$ in H_2O/MeCN (85:15)].

The *Klebsiella oxytoca* hydrolase [19,20], which was originally developed for production of (*S*)-α-arylpropionic acid via enantioselective hydrolysis of its methyl ester in a racemic form [14,21,22], shows some intriguing properties worth mentioning from a viewpoint of its industrial application: It shares little, if any, homology of the amino acid sequence with any known lipases, proteases, or esterases. The hydrolase is now available in quantity since its gene has been cloned and overexpressed successfully in engineered *E. coli* cells to achieve 600 times higher productivity than in the original *Klebsiella oxytoca* strain [20]. The hydrolase can function at temperatures as high as 70 °C while its producer, *Klebsiella oxytoca*, cannot survive such high temperatures; and it was such thermal stability that helped develop scalable processes to access (*R*)-BocDMTA **1** as discussed below [11].

With the hydrolase acting on (±)-**6b** in the desired stereochemical sense and excellent selectivity, an attempt was made to increase the concentration of (±)-**6b** beyond 1 M, thereby to make the enzymatic process scalable and industrially viable: When the enzymatic hydrolysis was conducted at 40 °C, the concentration of (±)-**6b** could be increased up to 1 M with no deleterious effect on its enantioselectivity or conversion rate. However, with further increase in the substrate concentrations over 1 M, the reaction mixture turned too viscous to be stirred even at 40 °C. Thus, we chose to take advantage of the thermally stable nature of the enzyme: when heated to 60 °C, a 3 M aqueous mixture of (±)-**6b** was found to be well stirred and (*R*)-**6a** was generated in 98% ee at 35% conversion with the use of the enzyme at a 0.3% (w/v) concentration.

Ultimately, a 3 M (575 g/L) aqueous mixture of (±)-**6b**, prepared in quantity from (±)-penicillamine **5**, was treated with the *Klebsiella oxytoca* hydrolase (3 g/L) at 60 °C for 26 h until the enzymatic hydrolysis reached 34% conversion (Scheme 1). The spent mixture was basified and extracted with *t*-butyl methyl ether (MTBE) to recover the left-over (*S*)-ester **6b** in 50% ee [Chiralpak AD (Daicel); *n*-hexane/*i*-PrOH/Et_2NH (75:25:0.3)].



Scheme 1. Scalable chemoenzymatic preparation of (*R*)-BocDMTA **1** using a thermally stable *Klebsiella oxytoca* hydrolase [11].

For (*R*)-acid **6a** digested in 97% ee ($E = 145$), its isolation was attempted but to no avail due to its amphoteric water-soluble nature. Hence, to the basic aqueous phase dissolving (*R*)-acid **6a** was added *i*-PrOH followed by $(Boc)_2O$ to form carbamate on the spot. Finally, extraction with AcOEt followed by single recrystallization from *n*-heptane–AcOEt afforded (*R*)-BocDMTA **1** in 24% overall yield from (±)-**6b**, the enantiomeric purity of (*R*)-**1** being determined to be 99.4% ee by chiral HPLC analysis [Chiralcel OD (Daicel); *n*-hexane/*i*-PrOH (98:2)] of its derived methyl ester (10% *n*-hexane solution of Me_3SiCHN_2, MTBE).

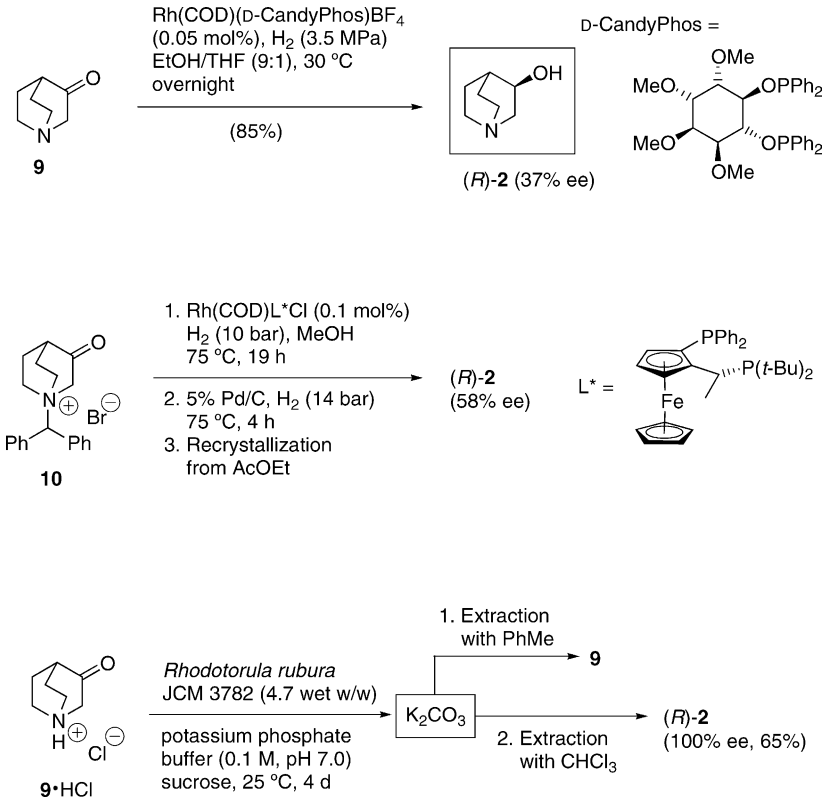
In the meantime, the left-over (*S*)-ester **6b**, which had been extracted into MTBE, was treated with a catalytic amount of NaOMe in PhMe to regenerate (±)-**6b** in 46% overall yield from the virgin (±)-**6b**, thereby replenishing the racemic substrate for another round of the enzymatic resolution.

3. Enzymatic resolution to access (*R*)-3-quinuclidinol 2: capturing cleaved carboxylic acid helps restore the enzymatic activity

Serving as a common pharmacophore of muscarinic receptor ligands, (*R*)-**2** has been required to build new drug candidates under clinical investigation, such as talsaclidine **7** (M_1 agonist; cognition enhancer) [23] and revatropate **8** (M_3 antagonist; bronchodilator) [24] (Fig. 2). To meet such pharmaceutical need, a scalable approach to (*R*)-**2** was developed by adapting a protease-mediated enantioselective hydrolysis for industrially viable processes [12].

To verify that the completed processes were worth exploring, prior tactics adopted to access (*R*)-**2** would rather be discussed in a comparative fashion. Reportedly, resolution of (±)-**2** via diastereomeric salt formation was attempted two-fold so far: one was to prepare a diastereomeric salt of (*R*)-**2** with (*S*)-camphorsulfonic acid in a solution of *i*-PrOH/acetone [25]; and the other was to combine *N*-benzyl (±)-3-hydroxyquinuclidinium chloride with a semi silver salt of dibenzoyl D-tartaric acid in water [26]. However, each endeavor ended up less amenable to scale-up: the former method afforded the diastereomeric salt in a marginally low overall yield of 6% after two recrystallizations [25]; and the latter method required extra steps such as preparation of the semi-silver-salt resolving agent from sodium hydrogen dibenzoyl D-tartrate and $AgNO_3$, *N*-benzylation of (±)-**2** prior to the diastereomeric salt formation, and hydrogenolytic removal of the *N*-benzyl group after the resolution [26].

As regards asymmetric reduction of 3-quinuclidinone **9**, its prochirality proved too subtle to be discerned by chiral organometallic catalysts, whether its bridgehead nitrogen was blocked by a bulky group or not, as indicated by the mediocre results reported to date (Scheme 2): Catalytic hydrogenation of **9** over a rhodium complex bearing chiral C_2-symmetric diphosphinite dubbed D-CandyPhos provided (*R*)-**2** in 37% ee [27]. When



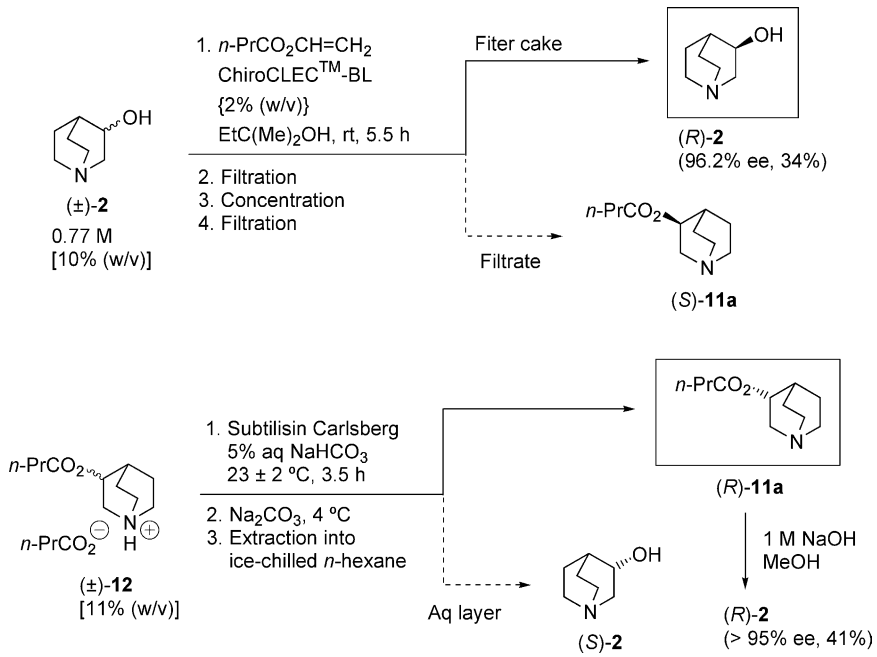
Scheme 2. Preparation of (*R*)-3-quinuclidinol **2** via asymmetric reduction using chiral rhodium complexes [27,28] and microbial cells [32].

N-benzhydryl 3-oxoquinuclidinium bromide **10** was subjected to catalytic hydrogenation over a chiral ferrocenyl diphosphine-modified rhodium complex, (*R*)-**2** was obtained in 58% ee after removing the *N*-benzhydryl group via palladium-catalyzed hydrogenolysis [28].

To overcome such difficulty in the enantiotopic face-discrimination with bicyclic ketone **9** and derivatives thereof, microbial reduction was explored in a few laboratories [29–31] including ours [32]: A homogeneous mixture of 3-quinuclidinone hydrogen chloride **9**·HCl {3.2% (w/v)} and a potassium phosphate buffer (KPB) solution (pH 7.0, 0.1 M) was treated with cells of *Rhodotorula rubra* JCM 3782 in 4.7 times the weight of the substrate for 4 days, and optically pure (*R*)-**2** could be extracted into $CHCl_3$ in 65% yield under basic conditions (K_2CO_3) after the unconsumed ketone **9** had been removed by PhMe-extraction (Scheme 2) [32]. Such excellent enantioselectivity and facile product isolation notwithstanding, the microbial reduction seems to have a long way to go before its adaptation for industrial production of (*R*)-**2** since it suffered from not only the low substrate concentration but also the mediocre yield.

In view of the less than satisfactory track record in preparation of (*R*)-**2** via diastereomeric salt formation and asymmetric reduction as well, attention was directed towards hydrolase-mediated kinetic resolution of (±)-**2** and a literature search uncovered two applications of a *Bacillus licheniformis* protease called subtilisin to the kinetic resolution in question. According to Lonza's patent application [33], cross-linked microcrystals of subtilisin (ChiroCLEC^{TM}-BL) [34] were reported to catalyze (*S*)-selective esterification of (±)-**2** with vinyl butyrate in *tert*-amyl alcohol (Scheme 3) whereby (*R*)-**2**, which survived the protease-catalyzed esterification unaffected, was obtained in 96.2% ee and 34% yield on precipitation from the spent mixture followed by single recrystallization. Cross-linked enzyme crystals being the cutting-edge of modern enzyme technologies [34], however, the substrate concentration of 10% (w/v) seems too low for the enzymatic esterification to attain industrially acceptable throughput. Another application of subtilisin reported by Muchmore at Bend Research involved (*S*)-selective hydrolysis of (±)-3-butyryloxyquinuclidinium butyrate **12** (Scheme 3) [35]: When an aqueous solution of (±)-**12** {11% (w/v)} was treated with subtilisin itself, (*S*)-selective hydrolysis took place leaving the (*R*)-ester **11a** unaffected, which was extracted into ice-chilled hexane selectively. Finally, methanolysis followed by crystallization provided (*R*)-**2** in >95% ee in 41% overall yield.

Muchmore's procedures being plagued with the less practical substrate concentration of 11% (v/w) [35], enzymatic hydrolysis in aqueous media would provide two practical advantages over enzymatic esterification in organic media: (1) a crude enzyme preparation can be used more conveniently in water, thereby dispensing with protein purification or immobilization, which often ends up costly and laborious; (2) with enzymatic hydrolysis proceeding in wa-



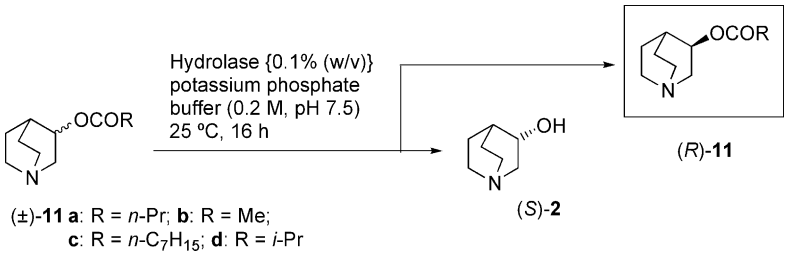
Scheme 3. Preparation of (*R*)-3-quinuclidinol **2** via subtilisin-mediated enantioselective esterification [33] and hydrolysis [35].

ter, usage of organic solvents can be reduced to champion the cause of green chemistry.

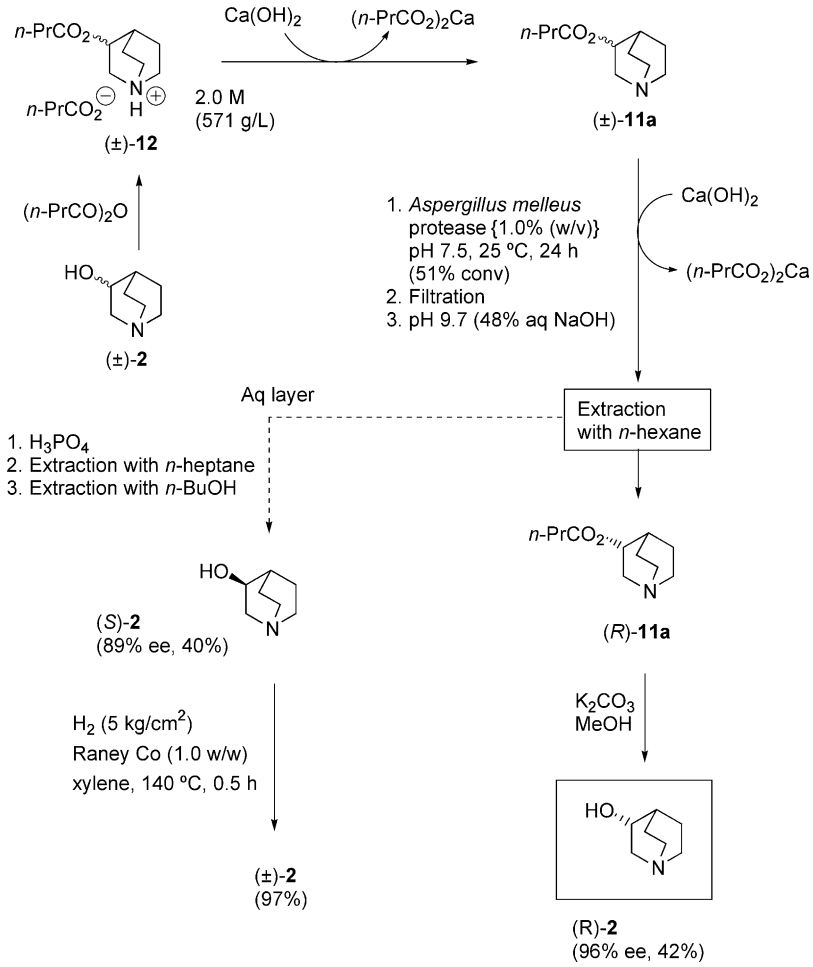
On the basis of these considerations, the enzymatic hydrolysis of racemic esters of (±)-**2** was explored for scalable processes to access (*R*)-**2** [12]: When commercially available enzymes {0.1% (w/v)} were tested for the (*S*)-selective hydrolysis of (±)-3-quinuclidinyl butyrate **11a** (0.2 M; prepared from (±)-**2** under the standard conditions) in a KPB solution (0.2 M, pH 7.5) at 25 °C (Scheme 4), an *Aspergillus melleus* protease (XP-488; available from Nagase ChemteX Corporation) showed the best mix of enantioselectivity and conversion rate [75% ee for (*R*)-**11a**, 95% ee for (*S*)-**2**; $E = 98$ at 44% conversion after 16 h] as monitored by HPLC analysis [conversion: CAPCELL PAK C-18 (Shiseido), MeOH/2.5% aqueous NH_3 (45:55); ee for (*R*)-**11a**: Chiralcel OD (Daicel), *n*-hexane/*i*-PrOH/CF_3CO_2H (90:10:0.1)] [12]. To uncover the fittest substrate for the newly identified protease, ester analogs such as (±)-**11b**–**d** were also prepared and each of them was subjected to the above-mentioned screening conditions (Scheme 4): [*A. melleus* protease] = 0.1% (w/v), a 0.2 M KPB solution (pH 7.5), 25 °C, 16 h [12]. Among the substrates tested, (±)-butyrate **11a** (R = *n*-Pr) proved to be the substrate of choice, exhibiting the greatest *E* value along with a balanced conversion rate: $E = 98$ at 44% conversion and 75% ee for (*R*)-**11a**.

Favoring medium-sized straight-chained esters such as butyrate **11a**, the *Aspergillus melleus* protease showed an interesting trend in terms of its substrate preference (Scheme 4): For the chain length of the esters, the protease was tolerant enough to accommodate eight-carbon straight-chained caprylate **11c** [R = *n*-C_7H_{15}; $E = 67$ at 40% conversion, 94% ee for (*R*)-**11c**], while acetate **11b** (R = Me) underwent little hydrolysis (2% conversion after 16 h). In contrast, the protease was so susceptible to the α-branching in the ester moiety that it could hydrolyze isobutyrate **11d** (R = *i*-Pr) only reluctantly [$E = 70$ at 28% conversion, 94% ee for (*R*)-**11d**].

With the ester structure fittest to the protease in hand, experimental effort was made to improve the enzymatic process in terms of operational simplicity and volume efficiency (Scheme 5) [12]. To telescope the substrate preparation into the enzymatic hydrolysis, Muchmore's procedures [35] were adopted whereby (±)-**2** was added neat to butyric anhydride and the resulting (±)-3-butyryloxyquinuclidinium butyrate



Scheme 4. (*S*)-Selective hydrolysis of esters of (±)-3-quinuclidinol **11** [12].



Scheme 5. Preparation of (*R*)-3-quinuclidinol **2** via *Aspergillus melleus* protease-mediated enantioselective hydrolysis and racemization of (*S*)-**2** over Raney Co [12].

12 was subjected directly to the protease-catalyzed hydrolysis. When a 1 M aqueous mixture of (±)-**12** was treated with the protease at a 0.5% (w/v) concentration at 25 °C and the pH of the reaction was adjusted to 7.5 with sodium hydroxide in place of the KPB solution (0.2 M, pH 7.5), the enzymatic hydrolysis proceeded to give the left-over (*R*)-ester **11a** in 94% ee at 50% conversion after 24 h uneventfully. However, when molar concentration of the substrate approached 2 M with its ratio to the concentration of the protease {% (w/v)} being kept constant at 2:1, which was equivalent to 1 M for [(±)-**12**] versus 0.5% (w/v) for [*A. melleus* protease], the enzymatic hydrolysis was found to suffer significant decrease in enantioselectivity and slight retardation; in fact, (±)-**12** at concentration of 2.0 M underwent the protease-catalyzed hydrolysis giving (*R*)-**11a** in 78% ee at 45% conversion after 24 h [12].

These unfavorable phenomena seems to arise from product inhibition caused by an incredibly high concentration of butyric acid, which was introduced to the reaction medium as part of racemic substrate (±)-**12** and then built up with the progress of the enzymatic hydrolysis. Thus, several inorganic bases were tested for the ability to alleviate such inhibition by capturing butyric acid and squeezing it from the reaction medium. As a result, intensive experimentation led to identification of $Ca(OH)_2$ as so effective a scavenger as to keep the concentration of butyric acid below a harmless level; the substrate concentration could be increased successfully to 2 M (571 g/L) with the help of $Ca(OH)_2$ in keeping the pH of the reaction at 7.5.

In the event, scalable procedures for the protease-mediated kinetic resolution of (±)-**2** were established as follows (Scheme 5) [12]: (±)-3-Butyryloxyquinuclidinium butyrate **12**, obtained by adding (±)-**2** neat to butyric anhydride, was dissolved in water to prepare a 2 M aqueous solution of (±)-**12**. To the homogeneous mixture was added solid $Ca(OH)_2$ to adjust its pH to 7.5 and the *A. melleus* protease {1 % (w/v)} was added to set off the enzymatic reaction. Butyric acid being formed with the progress of the hydrolysis was then neutralized with solid $Ca(OH)_2$ to keep the pH of the reaction at 7.5, which afforded the left-over (*R*)-ester **11a** in 96% ee at 51% conversion after 24 h [12]. To separate the left-over (*R*)-ester **11a** from the digested (*S*)-alcohol **2**, their difference in hydrophobicity was used to advantage [12]: After solid precipitates of $Ca(OCOPr\text{-}n)_2$ were removed by filtration, the filtrate was basified to pH 9.7 and the more lipophilic (*R*)-ester **11a** was selectively ex-

tracted into *n*-hexane. Finally, basic methanolysis (K_2CO_3) afforded (*R*)-3-quinuclidinol **2** in 96% ee and 42% overall yield from (±)-**2** uneventfully.

The aqueous layer was acidified with phosphoric acid such that all the residual butyric acid was freed and extracted into *n*-heptane. Extraction with *n*-butanol provided (*S*)-**2** in 89% ee and 40% overall yield from (±)-**2** after purification via formation of the hydrochloride salt (*S*)-**2**·HCl of high crystallinity.

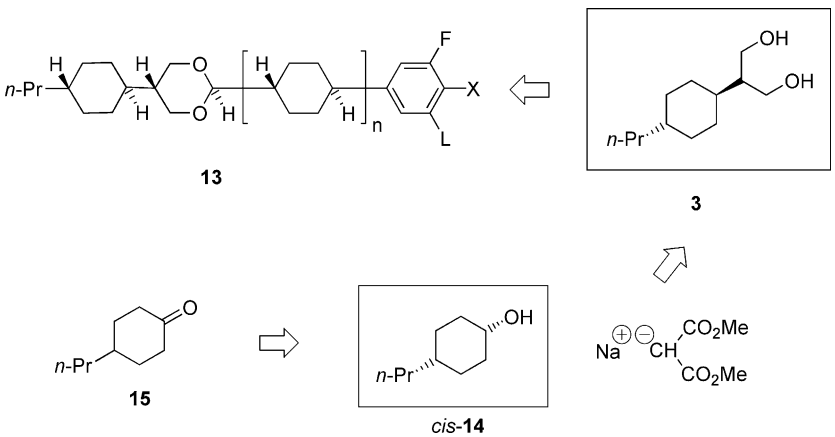
As regards the unwanted (*S*)-**2**, it was urgently needed to develop a practical method to recycle it since its racemic progenitor (±)-**2** is too precious a material to waste, the literature synthesis of the racemate requiring as many as five steps starting from ethyl isonicotinate [25,36]. Its stereogenic center being located on an isolated secondary carbinol carbon, (*S*)-**2** should be difficult to racemize by means other than concomitant oxidation and reduction [37]. Raney alloys were tested for the racemization, and the most effective recipe was found consisting of Raney cobalt, hydrogen and xylene [12]: when a xylene suspension of (*S*)-**2** of 89% ee and the same weight of Raney cobalt was heated to 140 °C under hydrogen at 5 kg/cm^2 pressure, the racemization went to completion in half an hour as monitored by GLC [β-DEX (Sperco), He (200 kPa), 140 °C] and (±)-**2** was regenerated in an isolated yield of 97% (Scheme 5). In the meantime, (*S*)-**2** was treated with an excess amount of Raney cobalt at 142 °C in the absence of hydrogen as a control experiment, and 3-quinuclidinone **9** was found to be generated in 97% conversion after 10 h, which underpinned the oxidation/reduction mechanism underlying the racemization process [12].

4. Chemoenzymatic synthesis of *trans*-1-(1,3-dihydroxypropan-2-yl)-4-propylcyclohexane 3: microbial diversity enables equatorial hydride delivery

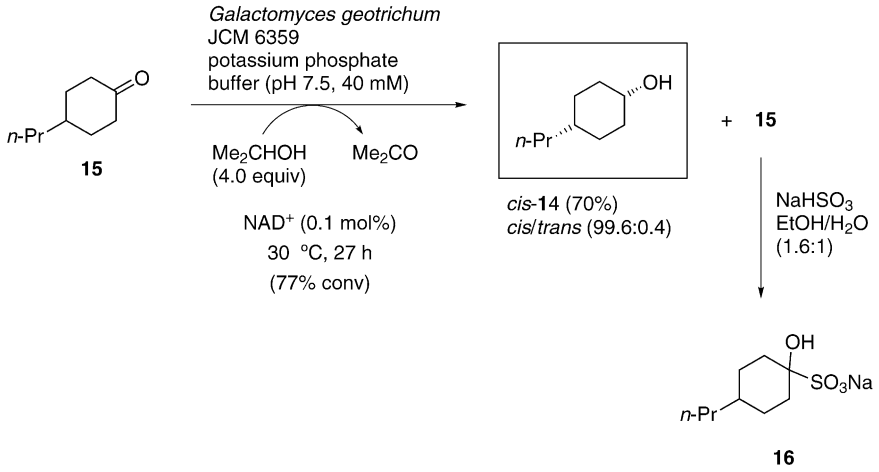
trans-1-(1,3-Dihydroxypropan-2-yl)-4-propylcyclohexane **3** [38] is a common immediate precursor to certain liquid crystal components represented by the general formula **13** (Scheme 6) [39,40]. Thus, it was envisioned that 1,4-*trans* disubstituted cyclohexane **3** could, in turn, be assembled from *cis*-4-propylcyclohexanol **14** by a classical malonate chemistry with stereochemical inversion. To implement such a synthetic plan, a practical scalable means was required which could allow 4-propylvyclohexanone **15** to be reduced to *cis*-alcohol **14** by equatorial hydride delivery.

However, building an axial alcohol in the reduction of 4-alkylcyclohexanone is a classical issue of stereochemistry that still remains to be addressed in industry. Indeed, to reduce 4-*tert*-butylcyclohexanone to *cis*-4-*tert*-butylcyclohexanol, bulky hydride agents can be used in the laboratory: Li[*t*-Bu(Et)$_2$CO]$_3$AlH in THF at 20 °C, *cis*/*trans* (95:5) [41]; Li[Me$_2$CH(Me)CH]$_2$BH in THF at −78 °C, *cis*/*trans* (>99.5:<0.5) [42]; and Li[2,4-(*t*-Bu)$_2$C$_6$H$_3$O](*t*-BuCH$_2$O)AlH in THF/Et$_2$O at 25 °C, *cis*/*trans* (64:5) [43]. However, their use in industry would be restricted due to their limited availability and safety concerns arising from their pyrophoric nature. Apart from such modified hydride agents, catalytic hydrogenation (1 atm, 25 °C) over rhodium in the presence of HCl in *i*-PrOH was also reported to be effective for the *cis*-selective reduction of 4-*tert*-butylcyclohexanone to give *cis*-4-*tert*-butylcyclohexanol in a *cis*/*trans* ratio of 99.3:0.7 [44]. However, hydrogenation under acidic conditions is less amenable to scale-up because usual plant hydrogenation facilities are not glass-lined and as such, tend to rust on exposure to acid. Therefore, neither chemical reduction of 4-alkylcyclohexanone would help produce *cis*-4-alkylcyclohexanol, such as 4-*cis*-propylcyclohexanol **14**, in quantity.

Actually, the case was the same with microbial reduction since no *cis*-selective reduction of 4-alkylcyclohexanone was reported so far [45,46]. Despite such a negative track record, the microbial world seems worth exploring because of its huge diversity; and this optimistic hypothesis was rewarded when a properly designed screening program was implemented with patience and diligence as discussed below. When about 800 microbes were tested for the *cis*-selective



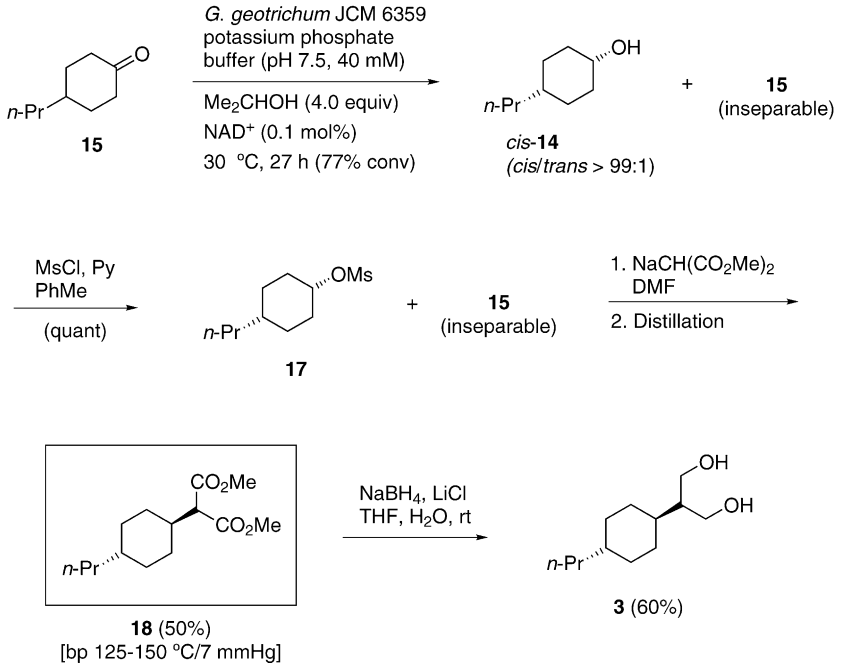
Scheme 6. Structures of liquid crystal components **13** [39,40] and their common synthetic precursor **3** [38] and a projected approach to **3** via *cis*-4-propylcyclohexanol **14** [47].



Scheme 7. *Galactomyces geotrichum*-mediated reduction of 4-propylcyclohexanone **15** to *cis*-4-propylcyclohexanol **14** [13].

reduction of 4-propylcyclohexanone **15** in the presence of adequate amounts of reduction cofactors such as NADH and NADPH, a yeast called *Galactomyces geotrichum* was identified as the biocatalyst of choice after a three-stage screening campaign: (1) search for microbes able to reduce **15** (4 g/L) in the presence of both NADH (0.5 equiv.) and NADPH (0.5 equiv.) in 0.1 M KPB (pH 7) in 2 h conversion >40% with the *cis*/*trans* selectivity >4:1 [GLC: TC-WAX (GL Science), He (52 kPa), 150 °C]; (2) search for microbes possessing intrinsically high reducing potential as demonstrated by the ability to reduce **15** (13 g/L) in 2 h conversion >25% with the *cis*/*trans* selectivity >4:1 in the presence of either NADH (1.0 equiv.) or NADPH (1.0 equiv.) in 0.1 M KPB (pH 7.0) along with identification of the nicotinamide cofactor preferred by each microbe; (3) search for auxiliary substrates to recycle the nicotinamide cofactor (0.01 equiv.) with the select microbes able to reduce **15** (60 g/L) in 20 h conversion >60% with the *cis*/*trans* selectivity >4:1 in 0.1 M KPB (pH 7.0) in the presence of test auxiliary substrates (each 2.0 equiv.), such as sugars, alcohols, α-amino acids, α-hydroxy acids, and formic acid.

In the event, the optimum conditions for the microbial reduction were established as follows (Scheme 7) [13]: *G. geotrichum* cells that had been grown in a YM medium at 30 °C for 2 days were harvested by centrifugation and they were mixed with a heterogeneous mixture of ketone **15** (60 g/L), *i*-PrOH (4.0 equiv.), NAD^+ (0.1 mol%) and a KPB solution (pH 7.5, 40 mM) at 30 °C. When the reduc-



Scheme 8. Synthesis of *trans*-1-(1,3-dihydroxypropan-2-yl)-4-propylcyclohexane **3** [13,47].

tion reached 77% conversion in 27 h, the mixture was extracted with MTBE. The MTBE extract was concentrated, and the residue was treated with an aqueous ethanol solution of $NaHSO_3$ to convert unconsumed ketone **15** into bisulfite adduct **16**, which was insoluble in organic solvents of low polarity. Finally, extraction with PhMe provided practically pure *cis*-4-propylcyclohexanol **14** in 70% yield.

On second thought, our ultimate goal was to assemble *trans*-1,4-disubstituted cyclohexane **3** but not to prepare **14**. Thus, it was examined whether crude *cis*-alcohol **14** contaminated with unconsumed ketone **15** could be carried over to the ensuing reaction without purification (Scheme 8) [13,47]. The crude product [*cis*-**14**/**15** (77:23)] arising from the *G. geotrichum*-mediated reduction was treated with MsCl and Py in PhMe to give mesylate **17** uneventfully in preparation for installing a carbon appendage via nucleophilic substitution [13,47]. However, mesylate **17** being inseparable from ketone **15** without the help of chromatography, removal of the entailed ketone was postponed until the next alkylation step. Mesylate **17** contaminated with ketone **15** was then treated with dimethyl sodiomalonate (3.0 equiv.) in DMF to give *trans*-alkylated product **18** while ketone **15** survived the malonate attack unaffected. When the crude product mixture was subjected to simple distillation, ketone **15** could be removed completely as a lower-boiling fraction to afford purified **18** in 50% overall yield. Finally, diester **18** (bp 125–150 °C/7 mmHg) thus obtained was reduced with $NaBH_4$ in the presence of LiCl at room temperature to provide *trans*-1-(1,3-dihydroxypropan-2-yl)-4-propylcyclohexane **3** in a pure crystalline state in 60% yield [13,47].

5. Conclusions and outlook

The most effective way that hydrolases are used in industry is to let them catalyze hydrolysis in water because hydrolases are unique in their ability to differentiate between enantiomers in water while organometallic catalysts for asymmetric acylation can work only in organic media [4,5]. Another merit of employing hydrolases in water is to practice green chemistry by reducing organic solvent usage. All in all, hydrolases will continue to find their own niche in the fine chemicals industry once volume efficiency can be increased somehow to an industrially viable level and products of enzymatic hydrolysis can be separated and isolated in a practical manner. Microbial reduction will also thrive on recognition of subtle difference in heterotopic faces [48] of carbonyl groups which modern exquisite chemical agents fail to discern, as demonstrated in the *cis*-selective reduction of 4-propylcyclohexanone **15** and in the (*R*)-selective reduction of 3-quinuclidinone **9** as well.

In the final analysis, it is important to ask two questions in developing scalable enzymatic processes: firstly, ask from a strategic viewpoint what real advantage biocatalysis can bring about over any chemical alternative; secondly, ask from a tactical viewpoint whether practical procedures can be devised to separate product(s) of an enzymatic reaction. When positive answers can be found simultaneously to both questions, projected chemoenzymatic processes can then be adapted for industrial production successfully [10,14].

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