Organic Process Research & Development **2003**, *7*, 289−296

Lecture Transcripts

There's No Industrial Biocatalyst Like Hydrolase: Development of Scalable Enantioselective Processes Using Hydrolytic Enzymes[1]

Yasuo Chikusa,[†,⊥] Yoshihiro Hirayama,[†] Masaya Ikunaka,*[,†] Toru Inoue,[†] Shunji Kamiyama,[†] Masafumi Moriwaki,[†] Yukifumi Nishimoto,[†] Fumiki Nomoto,[†] Kazuo Ogawa,[‡] Tomoyasu Ohno,[§] Koutaro Otsuka,[†] Akiko K. Sakota,[†] Naoki Shirasaka,[†] Atsuko Uzura,[†] and Kensaku Uzura[∥]

Research & Development Center, Nagase & Co., Ltd., 2-2-3 Murotani, Nishi-ku, Kobe, Hyogo, 651-2241 Japan, Bio/Fine Chemicals Division, Nagase ChemteX Corporation, 1-52 Osadano-cho, Fukuchiyama, Kyoto, 620-0853 Japan, Saitama Factory, Taiho Pharmaceutical Co., Ltd., 200-22 Toyohara Motohara, Kamikawa-cho, Kodama, Saitama 367-0241 Japan, and Technological R & D Laboratories, Taiho Pharmaceutical Co., Ltd., 200-22 Toyohara Motohara, Kamikawa-cho, Kodama, Saitama 367-0241 Japan

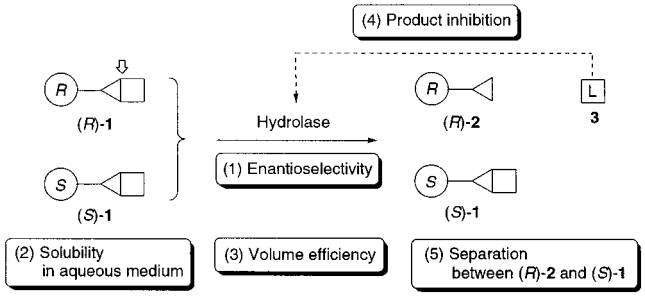
Abstract:

Chiral, racemic esters, ethyl (±)-tetrahydrofuran-2-carboxylate 4c, methyl (±)-α-phenylpropionate 9b, methyl (±)-5,5-dimethyl-1,3-thiazolidine-4-carboxylate 12a, 2-methoxyethyl (±)-1-(4-*tert*-butylphenyl)-2-oxopyrrolidine-4-carboxylate 15a, (±)-1-benzyloxy-3-chloropropan-2-yl hydrogen succinate 18c, and (±)-3-butyryloxyquinuclidinium butyrate [(±)-20b·*n*-PrCO$_2$H], are resolved kinetically by enantioselective hydrolysis catalyzed by an *Aspergillus melleus* protease [E = 60; 93.9% ee and 35% yield for (*R*)-tetrahydrofuran-2-carboxylic acid 4a], a *Klebsiella oxytoca* hydrolase [E > 200; 99.5% ee and 36% yield for (*S*)-α-phenylpropionic acid 9a], a *K. oxytoca* hydrolase [E = 145; 97.7% ee and 34% yield for (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid 12b], a *Bacillus brevis* protease [E = 77; 99% ee and 45% yield for (*S*)-15a], a *Serratia marcescence* esterase [E = 49; 99% ee and 43% yield for (*S*)-18c], and an *A. melleus* protease [E = 96; 96% ee and 42% yield for (*R*)-20b], respectively. Each enzymatic process is discussed with focus on the following tactical issues: (1) identification of a hydrolase with high enantioselectivity, (2) substrate concentrations not less than 1 M that allow for industrially viable volume efficiency (space−time yield), (3) product separation by partition between organic and aqueous phases, and (4) alleviation of a hydrolysate inhibiting the enzymatic activity.

Introduction

Because of their incomparable ability to discern molecular chirality, hydrolytic enzymes, such as lipases, proteases, and esterases, are now chiral catalysts indispensable for enantioselective synthesis.[2] However, contrary to a proven track record of hydrolases in the laboratory, there are few industrial processes benefiting from such hydrolase function.[3] What on earth brings about such a contradictory situation while hydrolase-catalyzed processes are assumed to be practical and amenable to scale-up?

Scheme 1. **Hydrolase-catalyzed kinetic resolution**



However simple enzymatic hydrolysis may appear, it is under the intricate influence of parameters itemized in Scheme 1 that illustrates kinetic resolution of a hypothetical racemic ester (±)-**1** by the catalysis of a hydrolase. For the kinetic resolution of (±)-**1** to be successful in industry, the following tactical issues should be addressed: (1) A hydrolase in hand should be able to digest one enantiomer, (*R*)-**1**, and leave the other, (*S*)-**1**, untouched with high enantioselectivity (allegedly E > 20).[4] (2) Its hydrophobicity (lipophilicity) notwithstanding, the organic substrate (±)-**1** should be put in contact with a hydrolase resident in an aqueous phase somehow effectively; heating or two-phase reaction

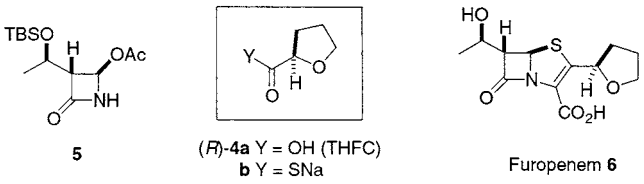
* To whom correspondence should be addressed. Telephone: +81 78 992 3164. Fax: +81 78 992 1050. E-mail: masaya.ikunaka@nagase.co.jp.
† Nagase & Co., Ltd.
‡ Saitama Factory, Taiho Pharmaceutical Co., Ltd.
§ Technological R & D Laboratories, Taiho Pharmaceutical Co., Ltd.
∥ Bio/Fine Chemicals Division, Nagase ChemteX Corporation.
⊥ Present address: Research & Development Division, Nagase ChemteX Corporation, 236 Tatsuno-nakai, Tatsuno, Hyogo, 671-4124 Japan.

(1) This is an enlarged and combined transcript based on two lectures: Stereoselective Synthesis of Chiral Pharmaceutical Intermediates (The Fourth International Conference on Organic Process Research and Development, Hong Kong, March 18−21, 2001) and Scalable Enantioselective Processes for Chiral Carboxylic Acids (The Fifth International Conference on the Scale-up of Chemical Processes, Jersey, U.K., September 23−26, 2002). For the other parts of the lectures and other papers at the conferences, contact Scientific Update, Maycroft Place, Stone Cross, Mayfield, East Sussex TN20 6EW, United Kingdom; Telephone: +44 1435 873062; Fax: +44 1435 872734; E-mail: sciup@scientificupdate.co.uk.

(2) Bornsheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis*: *Regio- and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999.
(3) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*; Wiley-VCH: Weinheim, 2000.
(4) (a) Bornsheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis*: *Regio- and Stereoselective Biotransformations*; Wiley-VCH: Weinheim, 1999; pp 32−33. (b) Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*; Pergamon: Oxford, 1994; pp 9−13.

10.1021/op034014b CCC: $25.00
Published on Web 04/09/2003

Chart 1. **Structures of furopenem 6 and its synthetic intermediates (*R*)-THFC 4a, 4b, and 5**



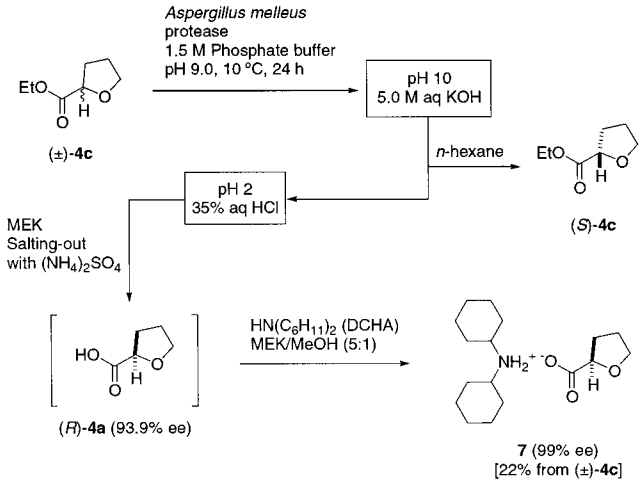
may be helpful, but is sometimes harmful to some hydrolases. (3) No industrially accepted volume efficiency (throughput, space-time-yield) would be achieved unless enzymatic hydrolysis could be run under far more condensed conditions than in the laboratory: [(±)-**1**] should be not less than 1 M. (4) A hydrolysate (L, **3**), whether it is alcohol or carboxylic acid, might inhibit the hydrolase activity, arresting the complete hydrolysis of (*R*)-**1**:[5] a phenomenon that is often overlooked in the laboratory where enzymatic hydrolysis can be conducted at low [(±)-**1**], and hence at low [L], in the presence of a heavy loading of the hydrolase. (5) Last but not least, separation between the digested (*R*)-**2** and the left-over (*S*)-**1** should be effected through partition (extractive workup) but not by chromatography.

The present communication will discuss select case studies on enzymatic enantioselective hydrolysis of chiral, racemic esters, shedding light on those methodological subjects mentioned above from a pragmatic perspective.

(*R*)-Tetrahydrofuran-2-carboxylic Acid (THFC) 4a: Mediocre Selectivity Leads to Compounded Results. The starter case study involves a protease-catalyzed kinetic resolution giving (*R*)-tetrahydrofuran-2-carboxylic acid (THFC) **4a** (Chart 1). (*R*)-THFC **4a** is a key intermediate to assemble furopenem **6** since its tetrahydrofuran ring has been incorporated into **6** via nucleophilic displacement on 4-acetoxyazetidinone **5** with sodium thioate (*R*)-**4b**.[6] Judging from its size and atomic composition, esters of (±)-THFC **4a** would be hydrophilic enough to be well dispersed in an aqueous phase and undergo enzymatic hydrolysis there. To the best of our knowledge, however, no enzyme-catalyzed enantioselective hydrolysis had been attempted to obtain (*R*)-THFC **4a** before we explored such possibilities. In fact, its production has depended only on resolution via diastereomeric salt formation.[7]

Hence, industrially available hydrolase preparations were tested for the enantioselective hydrolysis of esters of (±)-THFC **4a** including its methyl, ethyl, 2-propyl, benzyl esters, which nominated a combination of the ethyl ester of (±)-THFC **4a** [(±)-**4c**] and an *Aspergillus melleus* protease[8] for further investigation. Reaction parameters, such as substrate concentration, pH, buffer concentration, and temperature, were then explored to maximize both enantioselectivity and volume efficiency for the enzymatic process in question, which culminated in identification of the protocol depicted in Scheme 2:[9] (±)-**4c** (1 M, 144 g/L) was treated with the *A. melleus* protease (5 g/L) in a 1.5 M potassium phosphate buffer (pH 9.0) at 10 °C for 24 h. The spent mixture was basified to pH 10 with a 5.0 M aqueous KOH and extracted with *n*-hexane to recover the left-over (*S*)-ester **4c**. The aqueous layer was then acidified to pH 2 with 35% aqueous HCl, and the digested (*R*)-acid **4a** was extracted into methyl ethyl ketone (MEK); at the last stage of extraction, the aqueous layer was salted out with ammonium sulfate to allow complete recovery of (*R*)-**4a**, which was extremely elusive due to its high water solubility. When the MEK solution was analyzed by chiral HPLC [CHIRALPAK WH (Daicel), 2 mM aqueous $CuSO_4$], (*R*)-THFC **4a** of 93.9% ee was shown to be produced with $E = 59.9$ in 34.9% yield.

Scheme 2. ***A. melleus*** **protease-catalyzed kinetic resolution of the ethyl ester of (±)-THFC 4c**



Its optical purity being less than satisfactory, (*R*)-THFC **4a** thus generated was combined with an achiral organic base to form a crystalline salt in the expectation that its fractional crystallization would increase the optical purity of (*R*)-**4a** to 99% ee: The MEK solution of (*R*)-**4a** was concentrated in vacuo until the volume diminished to 16% of its original volume, and MeOH was added to adjust the MEK/MeOH ratio to 5:1. When *N*,*N*-dicyclohexylamine (DCHA, 1.0 equiv) was added to the solution, crystals of the DCHA salt of (*R*)-THFC **4a** (**7**) precipitated out in 22% overall yield from (±)-**4c** and in 99.1% ee as confirmed by the chiral HPLC analysis mentioned above.

Indeed, product purification via crystalline-salt formation did help complement the mediocre enantioselectivity of the

(5) For the inhibition caused not by a leaving group, such as L **3**, but by a resolved product, such as (*R*)-**2**, see: (a) Akeboshi, T.; Ohtsuka, Y.; Ishihara, T.; Sugai, T. *Adv. Synth. Catal.* **2001**, *343*, 624. (b) Lundhaug, K.; Overbeeke, P. L. A.; Jongejan, J. A.; Anthonsen, T. *Tetrahedron: Asymmetry* **1998**, *9*, 2851.

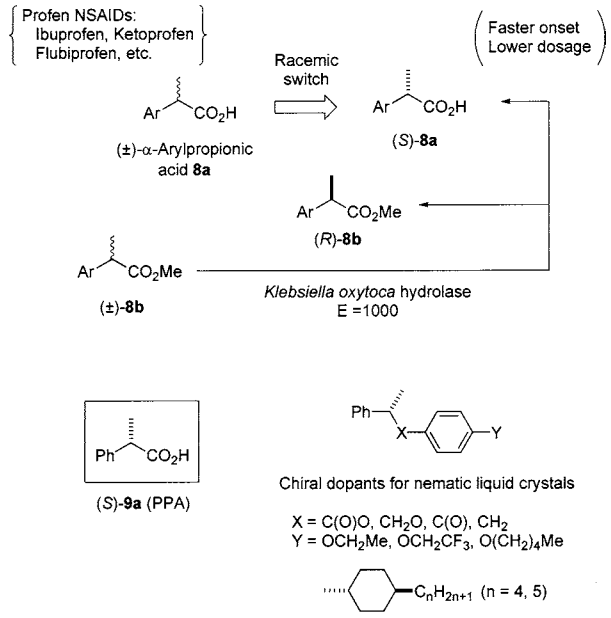
(6) (a) Nishino, T.; Maeda, Y.; Ohtsu, E.; Koizuka, S.; Nishihara, T.; Adachi, H.; Okamoto, K.; Ishiguro, M. *J. Antibiot.* **1989**, *42*, 977. (b) For a concise review on synthetic approaches to nonclassical β-lactam antibiotics, see: Carbi, W.; Di Fabio, R. *From Bench to Market: The Evolution of Chemical Synthesis*; Oxford University Press: Oxford, 2000; pp 25−47.

(7) (a) Bélanger, P. C.; Williams, H. W. R. *Can. J. Chem.* **1983**, *61*, 1383. (b) Červinka, O.; Bajanzulyn, O.; Fábryová, Šačuks, A. *Collect. Czech. Chem. Commun.* **1986**, *51*, 404. (c) Nohira, H.; Nagata, A.; Kondo, M.; Takebayashi, A. Jpn. Kokai Tokkyo Koho 89 216,983, 1989. (d) Nakai, S.; Sato, H.; Fujino, T. (Toray Industries, Inc.). Jpn. Kokai Tokkyo Koho 96 59,517, 1996. (e) van Eikeren, P.; McConville, F. X.; Lopez, J. (Sepracor, Inc.). WO 96/36,584, 1996.

(8) Available from Nagase ChemteX Corporation. Telephone: +81 773 27 3495. Fax: +81 773 27 6450. E-mail: kensaku.uzura@ncx.nagase.co.jp.

(9) (a) Nishimoto, Y.; Hirayama, Y.; Fujima, Y.; Ikunaka, M. (Nagase & Co., Ltd.). Jpn. Kokai Tokkyo Koho 2002 171,994, 2002. (b) For the influence of reaction parameters on both kinetics and enantioselectivity of the enzymatic reaction, see: Fujima, Y.; Hirayama, Y.; Ikunaka, M.; Nishimoto, Y. *Tetrahedron: Asymmetry* **2003**, *14*, in press.

***Scheme 3. K. oxytoca* hydrolase and (S)-α-arylpropionic acid 8a in industry**



hydrolase as demonstrated above; however, with a hydrolase exhibiting much higher enantioselectivity in hand, the enzymatic process would have ended up dispensing with such an extra step of purification. And the moral is as follows: *Festina lente (make haste slowly) when doing any enzyme screening since no chemical processes could compensate for the mediocre performance of a selected enzyme.*

(*S*)-α-Phenylpropionic Acid (PPA) 9a: Genetic Engineering Helps. Racemic switch is a program to redevelop a chiral, racemic drug already in clinical use into a single enantiomer drug of better pharmacological profiles, and it was early in the 1990s when a racemic switch of profen NSAIDs (nonsteroidal antiinflammatory drugs of the α-arylpropionic acid type) was in such vogue[10] that we set up a process R & D program to produce (*S*)-α-arylpropionic acid **8a**, the enantiomer possessing the intrinsic activity to inhibit cyclooxygenase, via enzyme-catalyzed enantioselective hydrolysis of its racemic methyl ester (±)-**8b** (Scheme 3).[11] Thus, not only stock cultures kept at Nagase ChemteX Corporation but also soil samples collected from the rural terrain in Japan were explored for microbes producing a hydrolase that was able to discriminate between the enantiomers of (±)-**8b** with excellent selectivity.

Such an extensive microbial screening eventually identified a *Klebsiella oxytoca* strain as a producer of a hydrolase that could catalyze the enantioselective hydrolysis of (*S*)-**8b** with an *E* value of no less than 1000.[12] However, it turned out that the *K. oxytoca* strain could produce the hydrolytic enzyme only in much less quantity than needed for further investigation into developing a scalable enzymatic resolution for (*S*)-**8a**. Hence, to secure an adequate supply of the hydrolase, the gene encoding it was cloned into pBluescript SK (+) and subcloned into pKK223-3 successfully to construct an expression vector (5.8 kb) bearing the exact ORF of the hydrolase.[13] *Escherichia coli* JM 105 was then transformed with the expression vector to have it to produce the hydrolase at productivity 600 times that of the original *K. oxytoca* strain, which has eventually enabled the industrial supply of the new hydrolase of the *K. oxytoca* origin.[14]

Quantities of the recombinant hydrolase in hand also allowed the enzymological study to reveal its unique properties: The *K. oxytoca* hydrolase has shared little, if any, homology with known lipases, esterases, or proteases. Another feature of more industrial importance is that it can still function at temperatures as high as 70 °C, and it was this feature of thermal stability that helped the chemoenzymatic processes for (*R*)-BocDMTA **10** attain such high volume efficiency as discussed in the next case study (Scheme 8).

In the meantime, however, the craze for the profen racemic switch diminished steeply after FDA disapproved the therapeutic advantage of (*S*)-ibuprofen [Ar = 4-isobutylphenyl for (*S*)-**8a**] over its racemate. In this aftermath, other synthetic programs were explored for application of the *K. oxytoca* hydrolase outside the pharmaceutical industry, which led to identification of (*S*)-α-phenylpropionic acid (PPA) **9a**, the simplest among the (*S*)-α-arylpropionic acid **8a** congeners, as a key intermediate to assemble those chiral dopants for nematic liquid crystals which are depicted to the right of (*S*)-PPA **9a** in Scheme 3 and as such, as a nominee for the *K. oxytoca* hydrolase-catalyzed kinetic resolution.[15]

When the methyl ester of (±)-PPA **9a** [(±)-**9b**] was treated with the *K. oxytoca* hydrolase, its (*S*)-enantiomer underwent selective hydrolysis as expected to give (*S*)-**9a** with high $E > 200$ as monitored by chiral HPLC analysis [CHIRALCEL OD (Daicel), *n*-hexane/*i*-PrOH/CF_3CO_2H (98:2:0.3 v/v/v)]. The reaction conditions were then explored for the optimal set of substrate concentration and hydrolase usage (Scheme 4): Eventually, it turned out that (±)-**9b** (3.34 kg, 1.0 M) could be processed with the *K. oxytoca* hydrolase (5.27 g) in a 0.5 M potassium phosphate buffer (pH 9). After 24-h stirring, the spent mixture was basified to pH 10.5 with 48% aqueous NaOH, and extracted with AcOEt to recover the unaffected (*R*)-**9b**. The aqueous layer was then acidified to pH 3.5 with 35% aqueous HCl. Extraction with *n*-hexane finally provided (*S*)-PPA **9a** (1.10 kg, 36%) in 99.5% ee and 99.7% chemical purity (HPLC, UV 254 nm), which could be integrated into electronics chemicals such as chiral LC dopants uneventfully.

From this case study can be drawn the following moral: *Genetic engineering is worth attempting if it can help*

(10) Stahly, G. P.; Starrett, R. M. Production Methods for Chiral Non-steroidal Anti-inflammatory Profen Drugs. In *Chirality in Industry II*: *Developments in the Commercial Manufacture and Applications of Optically Active Compounds*; Collins, A. N., Sheldrake, G. N., Crosby, J., Eds.; John Wiley & Sons: Chichester, 1997; pp 19−40.

(11) For the resolution of typical profens such as (±)-ibuprofen, (±)-ketoprofen, and (±)-naproxen into the respective (*S*)-enantiomers via diastereomeric salt formation with (*S*)-3-methyl-2-phenylbutylamine, see: Chikusa, Y.; Fujimoto, T.; Ikunaka, M.; Inoue, T.; Kamiyama, S.; Maruo, K.; Matsumoto, J.; Matsuyama, K.; Moriwaki, M.; Nohira, H.; Saijo, S.; Yamanishi, M.; Yoshida, K. *Org. Process Res. Dev.* **2002**, *6*, 291.

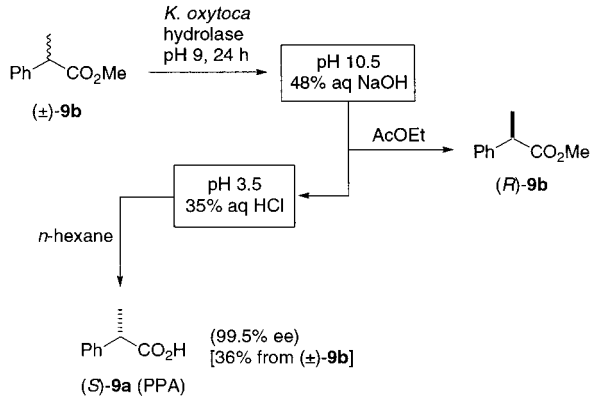
(12) Uzura, K.; Uzura, A.; Saegusa, M. (Nagase & Co., Ltd.). Jpn. Tokkyo Koho (Jpn. Patent) 3,093,039, 2000.

(13) Nomoto, F.; Kuramura, A.; Uzura, K. (Nagase & Co., Ltd.). Jpn. Kokai Tokkyo Koho 97 275,982, 1997.

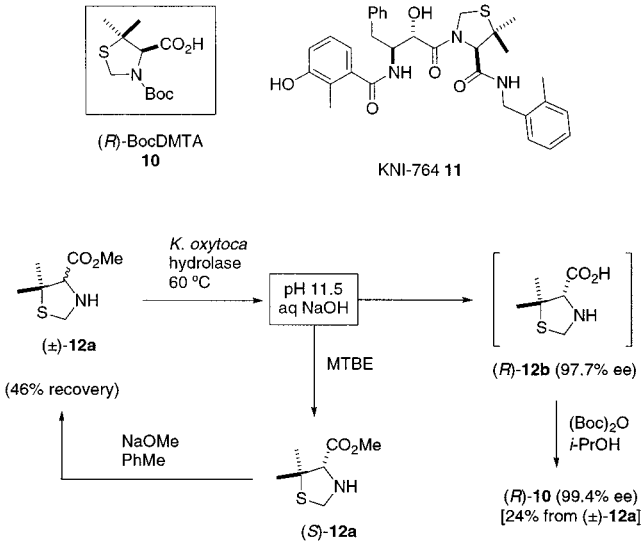
(14) For an available sample of the *K. oxytoca* hydrolase, contact the corresponding author.

(15) Nohira, H.; Aoki, Y.; Yokokoji, O.; Isono, K. (Seimi Chemical Co., Ltd.). Jpn. Kokai Tokkyo Koho 98 251,185, 1998.

Scheme 4. ***K. oxytoca*** **hydrolyase-catalyzed kinetic resolution of the methyl ester of (±)-PPA 9b**



Scheme 5. **Application of *K. oxytoca* hydrolase to chemoenzymatic synthesis of (*R*)-BocDMTA 10**



produce a unique enzyme which, otherwise, is difficult to obtain in sufficient quantities.

(*R*)-3-*tert*-Butoxycarbonyl-5,5-dimethyl-1,3-thiazoline-4-carboxylic Acid (BocDMTA) 10: Volume Efficiency Benefits from Enzyme's Thermal Stability. As part of a synthetic program to assemble chiral intermediates for HIV protease inhibitors in a practical enantioselective manner,[16,17] we explored chemoenzymatic approaches to (*R*)-3-(*tert*-butoxycarbonyl)-5,5-dimethyl-1,3-thiazoline-3-carboxylic acid (BocDMTA) **10**, a key intermediate for KNI-746 **11**, and attempted to resolve methyl (±)-5,5-dimethyl-1,3-thiazolidine-4-carboxylate **12a** kinetically by the enzyme-catalyzed enantioselective hydrolysis (Scheme 5).[17]

When 43 different hydrolase preparations were tested for the ability to differentiate between (*R*)- and (*S*)-**12a**, the *K. oxytoca* hydrolase was identified as the best catalyst that could perform such kinetic resolution ($E > 500$). To maximize the volume efficiency without compromising the enantioselectivity exerted, parameters were investigated which would affect enzymatic reactions in general: substrate concentration, enzyme usage, temperature, and reaction time. The bottom line was the thermal stability of the *K. oxytoca* hydrolase that enabled the reaction to run at temperatures as high as 60 °C where an aqueous suspension of (±)-**12a** could be stirred at a concentration of no less than 3 M. In fact, enzymatic hydrolysis proceeded with (±)-**12a** (3.0 M, 526 g/L) in the presence of the *K. oxytoca* hydrolase [0.3% (w/v)] at 60 °C and exhibited an *E* value of 145 at 34% conversion in 26 h, whereby (*R*)-**12b** was generated in 97.7% ee as determined by chiral HPLC analysis [SUMICHIRAL OA-5000 (Sumika), 2 mM solution of $CuSO_4$ in H_2O/MeCN (85:15 v/v)].

Running the enzymatic hydrolysis at 60 °C had another benefit: Being somewhat susceptible to MeOH, the *K. oxytoca* hydrolase may well suffer inhibition when (±)-**12a** undergoes hydrolysis at such high concentrations as mentioned above. Actually, however, no inhibition was observed at all as long as the reaction mixture was heated to 60 °C, where the released MeOH (bp 64 °C) should be evaporated to such an extent that its concentration was kept harmless to the hydrolase.

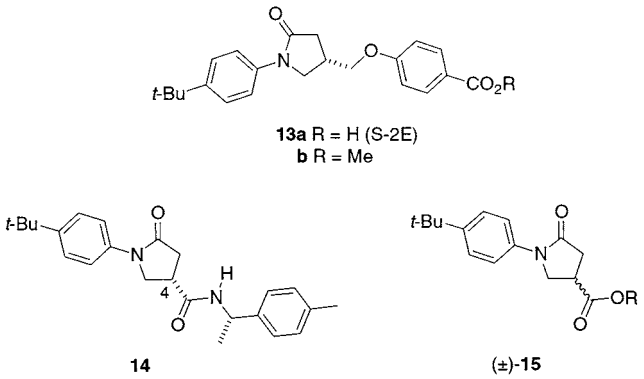
The hydrolyzed (*R*)-acid **12b** being extremely elusive due to its amphoteric nature as well as high water solubility, product isolation was postponed until its basic nitrogen functionality was blocked as a *tert*-butyl carbamate (Boc). Thus, the spent mixture was basified to pH 11.5 with aqueous NaOH and extracted with methyl *tert*-butyl ether (MTBE) to recover the left-over (*S*)-ester **12a** of 50.3% ee [chiral HPLC: CHIRALPAK AD (Daicel), *n*-hexane/*i*-PrOH/Et_2NH (75:25:0.3 v/v/v)]. The aqueous layer was diluted with *i*-PrOH such that the ensuing reaction would proceed under homogeneous conditions, and $(Boc)_2O$ was then added with ice-cooling. On completion of the carbamate formation, the volatile components were all removed by evaporation in vacuo, and the aqueous residue was acidified to pH 3.0 with aqueous HCl. Extraction with AcOEt followed by a single recrystallization [*n*-heptane/AcOEt (4:1 v/v)] eventually provided (*R*)-BocDMTA **10** of 99.4% ee in 24% overall yield from (±)-**12a**; the optical purity of (*R*)-**10** was determined by chiral HPLC analysis [CHIRALCEL OD (Daicel), *n*-hexane/*i*-PrOH (98:2 v/v)] of the corresponding methyl ester prepared by the Me_3SiCHN_2 treatment.[17]

To increase the effective yield of the whole chemoenzymatic processes for (*R*)-BocDMTA **10** beyond 50%, a theoretical upper limit imposed on any resolution, the (*S*)-ester **12a** that had survived the enzymatic hydrolysis was subjected to racemization to replenish the enzymatic substrate (±)-**12a**. When a solution of (*S*)-**12a** in PhMe was treated with a catalytic amount of NaOMe (28% solution in MeOH) for 4 h, (±)-**12a** could be regenerated in 46% overall yield from the virgin (±)-**12a**.

From the case study discussed above can be drawn the following moral: *A focused screening can identify a versatile enzyme* as demonstrated by the *K. oxytoca* hydrolase. While it was identified originally as a specific catalyst to selectively hydrolyze methyl (*S*)-α-arylpropionate **8b**, the versatility of the *K. oxytoca* hydrolase proved to be two-fold: (1) Its substrate specificity turned out tolerant enough to accommodate (±)-**12a** with little structural resemblance to (±)-**8b**

(16) Ikunaka, M.; Matsumoto, J.; Fujima, Y.; Hirayama, Y. *Org. Process Res. Dev.* **2002**, *6*, 49.
(17) Ikunaka, M.; Matsumoto, J.; Nishimoto, Y. *Tetrahedron*: *Asymmetry* **2002**, *13*, 1201.

Chart 2. **Structures of S-2E 13a and its chiral precursors 13b, 14, and (±)-15**



and differentiate between its enantiomers with high selectivity.[18] (2) It was able to function at temperatures as high as 70 °C, while its original producer, *K. oxytoca*, was classified as a mesophile growing favorably at temperatures between 20 and 50 °C.

2-Methoxyethyl (*S*)-1-(4-*tert*-butylphenyl)-2-oxopyrrolidine-4-carboxylate 15a: Microbes Work Wonders. S-2E **13a** is a potent hypolipidemic agent that can inhibit both cholesterol and fatty acid biosynthesis (Chart 2).[19] In the discovery synthesis of **13a**, a two-fold chiral separation was attempted:[19b] One was to separate the methyl ester of S-2E **13b** from its antipode by chiral chromatography [CHIRACEL OJ (Daicel), *n*-hexane/EtOH (4:1 v/v)]. The other was to separate *N*-[(*S*)-α-methyl-4-methylbenzyl]-(*S*)-1-(4-*tert*-butylphenyl)-2-oxopyrrolidine-4-carboxamide **14** from its (4*R*)-epimer by silica gel chromatography [$CHCl_3$/AcOEt (2:1 v/v)]. Since neither method seemed amenable to industrial production on scale, we embarked on exploring enzyme-catalyzed enantioselective hydrolysis of racemic ester (±)-**15**, keeping in mind that the parent (*S*)-acid of **14** had been converted to S-2E **13a** itself.[19b]

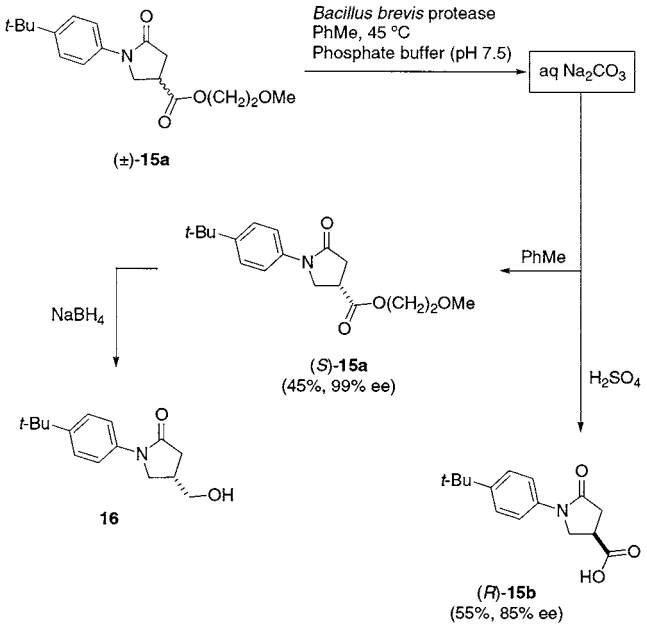
Industrially available hydrolase preparations were tested for the enantioselective hydrolysis of common esters of (±)-1-(4-*tert*-butylphenyl)-2-oxopyrrolidine-4-carboxylic acid **15** including its methyl ester [(±)-**15**, R = Me],[19a] and an *Aspergillus oryzae* protease[8] was identified as a nominee for further investigation into optimal conditions: When racemic ester (±)-**15** was treated with the *A. oryzae* protease, its (*S*)-enantiomer survived the hydrolysis, with its (*R*)-enantiomer undergoing selective hydrolysis. The sense of enantioselectivity in that the wanted (*S*)-**15** was left untouched was indeed favorable because (*S*)-ester **15** should be reduced to alcohol **16** (Scheme 6) much more easily in the next stage of the synthesis[19a] than its parent carboxylic acid.

Alcoholic residues of (±)-**15** were then explored for the improvement in not only enantioselectivity but also kinetics of the enzymatic hydrolysis, and 2-methoxyethyl ester (±)-**15a** (Scheme 6) [(±)-**15**, R = $(CH_2)_2OMe$ (Chart 2)]

(18) For other applications of the *K. oxytoca* hydrolase, see: (a) Kimura, M.; Kuboki, A.; Sugai, T. *Tetrahedron*: *Asymmetry* **2002**, *13*, 1059. (b) Kiyota, H.; Nakabayashi, M.; Oritani, T. *Tetrahedron*: *Asymmetry* **1999**, *10*, 3811.

(19) (a) Watanabe, S.; Ogawa, K.; Ohno, T.; Yano, S.; Yamada, H.; Shirasaka, T. *Eur. J. Med. Chem.* **1994**, *29*, 675. (b) Ohno, T.; Yano, S.; Yamada, H.; Shirasaka, T.; Yamamoto, A.; Kobayashi, K.; Ogawa, K. *Chem. Pharm. Bull.* **1999**, *47*, 1549.

Scheme 6. ***B. brevis*** **protease-catalyzed kinetic resolution of (±)-2-methoxyethyl ester 15a**



was identified as the substrate of choice. When this particular (±)-ester **15a** was treated with the *A. oryzae* protease, the hydrolysis proceeded with high enantioselectivity ($E = 63$) to give the left-over (*S*)-ester **15a** in 46% yield and 98% ee [chiral HPLC: OPTI-PAK TA (Waters), *n*-hexane/*i*-PrOH/CF_3CO_2H (70:30:0.1 v/v/v)].

No sooner had we started to explore reaction parameters to increase the volume efficiency than a catch-22 situation arose: With the concentration of (±)-**15a** approaching 1.0 M, the reaction mixture containing the *A. oryzae* protease (33 g/L) turned too viscous and gelatinous to be stirred even at 30 °C. Indeed, when the reaction temperature was increased beyond 30 °C, the mixture became less viscous to such an extent that stirring became possible again; however, with increasing temperature, the *A. oryzae* protease started to lose its activity, and the activity was lost completely around 40 °C.

What was worse, the *A. oryzae* protease turned out unfavorably susceptible to an organic solvent in which the substrate (±)-**15a** was dissolved. The reason that the hydrolase in use should be tolerant of the organic solvent is as follows: Being a rigid and water-insoluble solid, (±)-**15a** precipitated from the aqueous medium when added as it was. As such, (±)-**15a** had to be dissolved in an organic solvent, and the resulting solution added to the aqueous solution of the *A. oryzae* protease to ensure its dispersion in the reaction milieu. As a result, the mixture turned biphasic, and the *A. oryzae* protease was forced into contact with the organic solvent throughout the reaction.

To resolve all these contradictory problems, we chose to explore the local terrain for a microbe producing a new hydrolase that would outperform the *A. oryzae* protease in thermal stability and organic-solvent tolerance as well as enantioselectivity. After an intensive screening campaign, a *Bacillus brevis* strain was identified as producing a new protease that fulfilled all the criteria specified above:[20] The

enantioselectivity ($E = 77$) compared favorably with that of the *A. oryzae* protease ($E = 63$). The thermal stability did expand beyond 40 °C with no deterioration up to 50 °C. The *B. brevis* protease still retained its catalytic activity on prolonged exposure to the organic solvents used to dissolve (±)-**15a**, such as MEK, and PhMe. In addition, being a safe microorganism, the *B. brevis* strain was so easy to tame and grow that the protease became available in sufficient quantities to run the enantioselective hydrolysis of (±)-**15a** on an industrial scale (Scheme 6).[8]

For reference, the typical procedures taken in the laboratory are as follows:[20] A mixture of (±)-**15a** (16 g, 50 mmol) and PhMe (2 mL) was made homogeneous with heating and then added to a solution of the *B. brevis* protease preparation (1.5 g; lactose, 36%) in a 0.4 M potassium phosphate buffer (pH 7.5; 500 mL). The resulting biphasic mixture was stirred at 45 °C for 16 h. The spent mixture was basified to litmus alkali with 5% aqueous Na_2CO_3 and extracted with PhMe to provide (*S*)-**15a** in 45% yield and 99% ee [chiral HPLC: the same conditions as specified above]. When the aqueous layer was acidified to pH 2 with concentrated H_2SO_4, the digested (*R*)-acid **15b** was precipitated as solids in 55% yield and 85% ee [chiral HPLC: the same conditions as specified above].

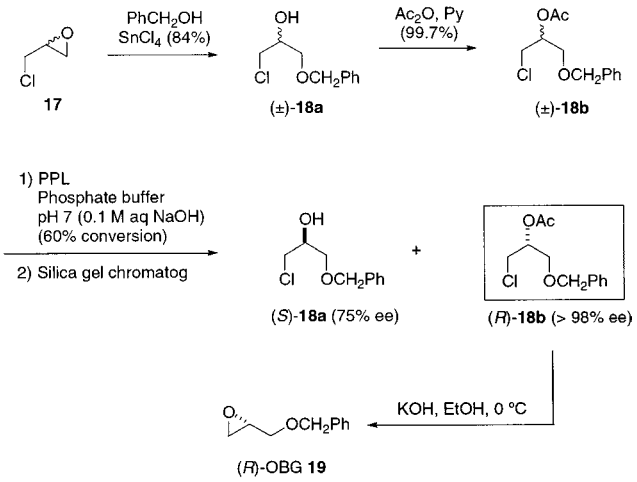
The (*S*)-ester **15a** thus obtained could be reduced to (*S*)-1-(4-*tert*-butylphenyl)-2-oxopyrrolidine-4-methanol **16**[19] with $NaBH_4$ uneventfully. The new and improved enzymatic process featuring the *B. brevis* protease merged now with the original drug-discovery route to S-2E **13a** itself. In the meantime, the enzymatic processes were modified and adapted successfully to multikilogram production of (*S*)-**15a**.

Microbial screening should exceed molecular biology-based methodology, such as directed evolution, in identifying a new enzyme with multifaceted improvements, as illustrated by the discovery of the *B. brevis* protease. That hydrolase which was superior three-fold to the *A. oryze* protease in enantioselectivity, thermal stability, and organic-solvent tolerance would not be obtained by other known methods than the focused and intensive screening. And the moral is as follows: *Spare the microbial screening and spoil any serendipitous success*.

(*R*)-*O*-Benzylglycidol (OBG) 19: Tethering Dissociable Functionality Makes Product Separation Practical. Being versatile chiral building blocks,[21] (*R*)- and (*S*)-*O*-benzylglycidol (OBG) **19** (Scheme 7) challenged synthetic chemists to develop their practical synthesis including chemoenzymatic approaches such as those reported by Wong et al.[22a] and Partali et al.;[22b] however, neither approach has met the practical requirements thus far.

For instance, Wong's pioneering work employed porcine pancreatic lipase (PPL) to hydrolyze (±)-2-acetoxy-1-benzyloxy-3-chloropropane **18b** enantioselectively, which had

Scheme 7. **Wong's chemoenzymatic synthesis of (*R*)-OBG 19**



been prepared uneventfully from (±)-epichlorohydrin **17** in two steps: (1) $SnCl_4$-catalyzed epoxide ring-opening with benzyl alcohol and (2) *O*-acetylation with Ac_2O in pyridine (Scheme 7).[22a] In the presence of PPL, (±)-**18b** went through enantioselective hydrolysis ($E = 33$ at 60% conversion) to give (*S*)-chlorohydrin **18a** in 75% ee, while (*R*)-acetate **18b** was left untouched in 98% ee; the optical purity of either compound was assessed with each acetate by chiral HPLC [CHIRALCEL OD (Daicel), *n*-hexane/*i*-PrOH (9:1 v/v)]. Silica gel column chromatography separated the left-over (*R*)-acetate **18b** from the digested (*S*)-alcohol **18a**. Possessing higher optical purity, the former was treated with KOH in EtOH at 0 °C to give (*R*)-OBG **19**, although neither single-step yield nor overall yield for it is specified in the literature.[22a]

Indeed, Wong's work should be praised for the first success in the kinetic resolution to access (*R*)-OBG **19**. However, it is plagued with one drawback: Both being neutral and similar in hydrophobicity, the digested (*S*)-alcohol **18a** and the left-over (*R*)-acetate **18b** should be so difficult to separate by partition that separation by silica gel column chromatography would be indispensable, detracting from its industrial viability. To overcome this separation problem, we chose to affix an extra dissociable functionality to the acetyl group of (±)-**18b**, expecting that it would allow the neutral digested alcohol only to be extracted into an organic phase with the unaffected ester remaining ionized and dissolved in an aqueous phase. Hence, racemic chlorohydrin (±)-**18a** was treated with succinic anhydride in pyridine to obtain (±)-1-benzyloxy-3-chloropropan-2-yl hydrogen succinate **18c** quantitatively.

Industrially available hydrolase preparations were then explored for the enantioselective hydrolysis of (±)-**18c**, which culminated in nominating a *Serratia marcescens* esterase[8] for further investigation into optimal conditions since it was able to leave (*S*)-**18c** untouched in the highest ee of any hydrolase investigated (Scheme 8).[23] Eventually, a scalable approach to (*S*)-OBG **19** was established which

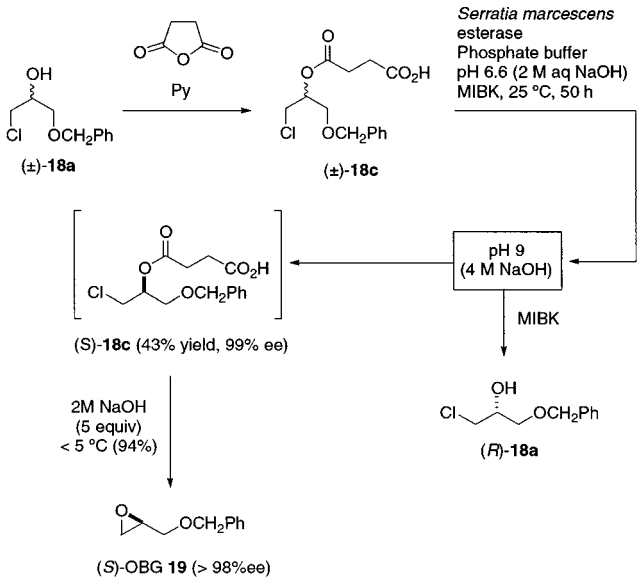
(20) Otsuka, K.; Kamiyama, S.; Moriwaki, M. (Taiho Pharmaceutical Co., Ltd. and Nagase & Co., Ltd.). Jpn. Kokai Tokkyo Koho 99 113,594, 1999.

(21) (a) Takano, S. *Yakugaku Zasshi* **1991**, *111*, 647. (b) Takano, S.; Sekiguchi, Y.; Setoh, M.; Yoshimitsu, T.; Inomata, K.; Takahashi, M.; Ogasawara, K. *Hetrocycles* **1990**, *31*, 1715.

(22) (a) Pedersen, R. L.; Liu, K. K.-C.; Rutan, J. F.; Chen, L.; Wong, C.-H. *J. Org. Chem.* **1990**, *55*, 4897. (b) Partali, V.; Waagen, V.; Alvik, T.; Anthonsen, T. *Tetrahedron: Asymmetry* **1993**, *4*, 961.

(23) Otsuka, K.; Yamamoto, C.; Umezato, T.; Kamiyama, S. (Nagase & Co., Ltd.). Jpn. Kokai Tokkyo Koho 97 140,394, 1997.

Scheme 8. ***S. marcescens*** **esterase-catalyzed kinetic resolution of hemisuccinate (±)-18c**



dispensed with silica gel column chromatography: To a 0.7% (w/v) solution of the *Serratia marcescens* esterase in a potassium phosphate buffer (pH 7) was added a 2 M solution of (±)-**18c** in methyl isobutyl ketone (MIBK), and the mixture was stirred at 25 °C for 50 h, during which the pH of the reaction was adjusted to 6.6 with 2 M aqueous NaOH. On completion of the enantioselective hydrolysis, the pH of the mixture was adjusted to 9 with 4 M aqueous NaOH. After the digested (*R*)-**18a** was extracted into MIBK completely, the aqueous layer was analyzed by chiral HPLC [CHIRALPAK AS (Daicel), *n*-hexane/*i*-PrOH/AcOH (97:3:0.3 v/v/v)] for the quantity and optical purity of the unaffected (*S*)-**18c**: 43% yield and 99% ee ($E = 49$). The aqueous layer was then treated with 2 M aqueous NaOH (5 equiv) below 5 °C to afford (*S*)-OBG **9** of >98% ee in 94% yield.

The tactical moral drawn from this case study is as follows: Prior to entering into experimentation, both substrate and product structures should be carefully examined to see if they can be separated from each other without recourse to chromatography. *If they are difficult to separate through partition, the enzymatic process in question should be redesigned, or another synthetic strategy devised.*[24]

(*R*)-Quinuclidin-3-ol 20a: Racemization Makes Enzymatic Resolution Perfect. Another case study on hydrolase-catalyzed kinetic resolution of chiral, racemic alcohol is concerned with enantioselective hydrolysis of an ester of (±)-quinuclidin-3-ol **20a**, its (*R*)-enantiomer **20a** serving as a common pharmacophore of neuromodulators acting on muscarine receptors,[25] such as revatropate **21** (M_3 receptor antagonist),[26] and talsaclidine **22** (M_1 receptor agonist)[27]

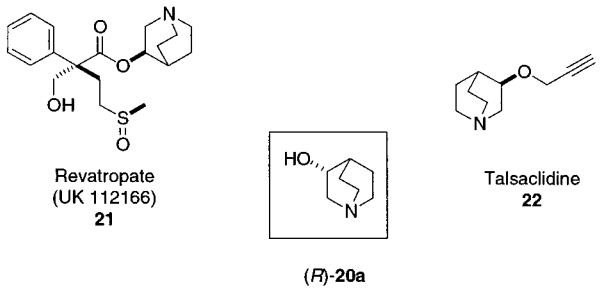
(24) For an ingenious design of enzymatic transesterification based on a related concept, see: Cantele, F.; Restelli, A.; Riva, S.; Tentorio, D.; Villa, M. *Adv. Synth. Catal.* **2001**, *343*, 721.

(25) Bjørsvik, H.-R.; Liguori, L.; Costantino, F.; Minisci, F. *Org. Process Res. Dev.* **2002**, *6*, 197.

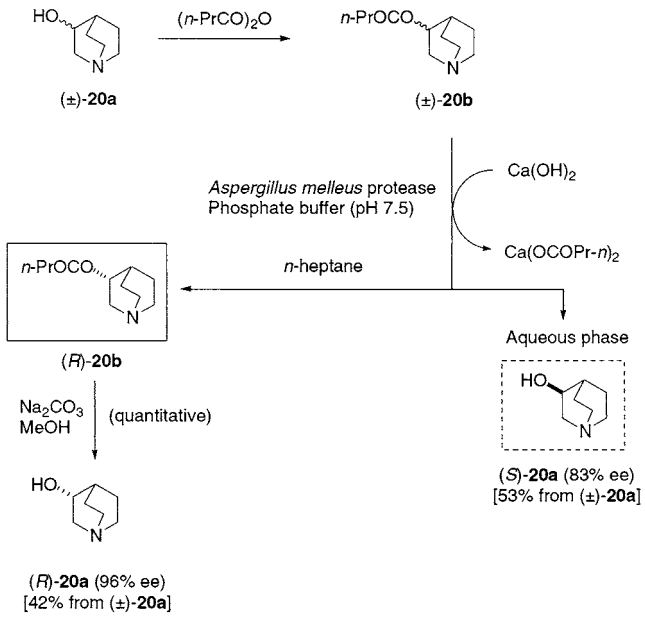
(26) Moore, B. A.; Keir, R. F.; Stuart, E. F.; Stobie, A.; Wright, K. N. *Eur. Respir. J.* **1996**, *9* (Suppl. 23), Abs. PO261.

(27) Leusch, A.; Tröger, W.; Greischel, A.; Roth, W. *Xenobiotica* **2000**, *30*, 797.

Chart 3. **Structures of (*R*)-quinuclidin-3-ol 20a, M_3 antagonist 21, and M_1 agonist 22**



Scheme 9. ***A. melleus*** **protease-catalyzed kinetic resolution of (±)-quinuclidin-3-yl butyrate 20b**



(Chart 3). (±)-Quinuclidin-3-yl butyrate **20b** was prepared from (±)-**20a** on treatment with butyric anhydride (Scheme 9), and industrially available lipases and proteases were tested for its enantioselective hydrolysis, which led to identification of an *Aspergillus melleus* protease[8] as the most promising catalyst: When (±)-**20b** (0.2 M) was treated with the *A. melleus* protease [0.1% (w/v)] in a 0.2 M potassium phosphate buffer (pH 7.5) at 25 °C, enantioselective hydrolysis proceeded to give (*S*)-quinuclidin-3-ol **20a** selectively, and (*R*)-quinuclidin-3-yl butyrate **20b** was left unaffected in 75% ee [CHIRALCEL OD (Daicel), *n*-hexane/*i*-PrOH/CF_3CO_2H (90:10:0.1 v/v/v)] with $E = 98$ at 44% conversion.[28] To further improve the enantioselectivity, structures of the carboxylic acid residues were varied, and butyrate of (±)-**20a** [(±)-**20b**] was confirmed to be the best substrate ($E = 98$) of any ester homologue investigated, such as the isobutyrate ($E = 70$), valerate ($E = 42$), and octanoate ($E = 67$).

For the combination of (±)-butyrate **20b** and the *A. melleus* protease, reaction parameters were next explored

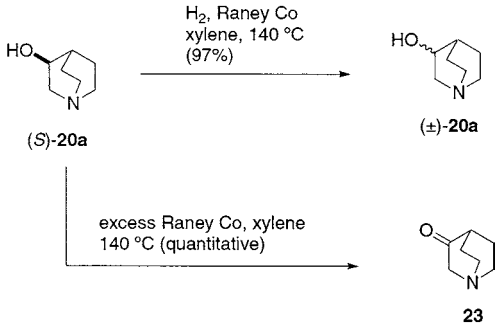
(28) (a) For a relevant approach using subtilisin Carlsberg, see: Muchmore, D. C. (Bend Research, Inc.). U.S. Patent 5,215,918, 1993. (b) *Spec. Chem. Mag.* **2001**, July/August, 14. (c) Nomoto, F.; Otsuka, K. (Nagase & Co., Ltd.). Jpn. Kokai Tokkyo Koho 98 210,997, 1998. (d) This subject will be discussed in depth with experimental details elsewhere: Nomoto, F.; Hirayama, Y.; Ikunaka, M.; Inoue, T.; Otsuka, K. *Tetrahedron: Asymmetry*. Manuscript submitted.

intensively for the optimum conditions in terms of conversion and volume efficiency (Scheme 9).[28d] The preliminary experimentation indicated that butyric acid released by the enzymatic hydrolysis inhibited the catalytic activity of *A. melleus* protease such that the progress of the hydrolysis was retarded. Thus, inorganic bases, including typical metal hydroxides, were tested for the ability to capture butyric acid and alleviate its inhibitory effect on the protease. Eventually, such screening efforts led to the identification of $Ca(OH)_2$ as the agent of choice to squeeze the liberated butyric acid out of the reaction milieu. When butyric acid was neutralized with $Ca(OH)_2$ as it was being generated by the enzymatic hydrolysis, the enantioselective hydrolysis did proceed with (±)-3-butyryloxyquinuclidinium butyrate [(±)-**20b**·*n*-$PrCO_2H$; 2.0 M, 571 g/L] in the presence of the *A. melleus* protease [1.0% (w/v)] to give the left-over (*R*)-butyrate **20b** in 96% ee at 42% conversion with $E = 96$ in 24 h.

Having established the scalable conditions for the enzymatic reaction itself, we turned our attention to the issue of product isolation, which was addressed in an empirical manner as follows:[28d] separation of the left-over (*R*)-butyrate **20b** from the digested (*S*)-alcohol **20a** was attempted by taking advantage of their difference in hydrophobicity (the tendency to be extracted into an organic solvent). On completion of the enzymatic hydrolysis, the spent mixture was extracted with a less polar organic solvent, such as heptane, to give the left-over butyrate (*R*)-**20b**, the more hydrophobic component, selectively in 42% yield. Extraction with a more polar solvent, such as $CHCl_3$, allowed the digested alcohol (*S*)-**20a**, the more hydrophilic component, to be recovered in 53% yield and 83% ee [chiral HPLC: the same conditions as specified above]. Finally, the (*R*)-butyrate **20b** thus obtained was subjected to methanolysis in the presence of Na_2CO_3 to provide (*R*)-quinuclidin-3-ol **20a** of 96% ee quantitatively, the three-step overall yield from (±)-**20a** being 42%.

Seemingly, the kinetic resolution of (±)-**20a** has ended in success; however, the resolution process as a whole would remain industrially less competitive unless the unwanted enantiomer (*S*)-**20a** is racemized for reuse, because (±)-**20a** is a rather expensive material.[29] Actually, however, its stereogenic center being an isolated secondary alcohol,

Scheme 10. **Racemization of (*S*)-quinuclidin-3-ol 20a**



racemization of (*S*)-**20a** was a more difficult task than that of (*S*)-**4c**, (*R*)-**8b**, (*R*)-**9b**, (*S*)-**12a**, and (*R*)-**15b**, each being susceptible to base-catalyzed racemization under the influence of the carboxyl group adjacent to the stereogenic center. Hence, we chose to explore oxidation/reduction procedures[30] for the racemization of (*S*)-**20a** (Scheme 10). When a xylene solution of (*S*)-**20a** was heated with Raney Co under an atmosphere of H_2 (5 kg/cm^2), racemization went to completion in half an hour to give (±)-**20a** in 97% yield, during which the progress of the racemization was monitored by chiral GLC [α-DEX 120 (Sperco), 140 °C].[31] The redox mechanism of racemization was then corroborated by the experimental fact that (*S*)-**20a** underwent quantitative oxidation to quinuclidin-3-one **23** when heated with excess Raney Co in the absence of H_2.

The morals drawn from this case study are two-fold: (1) *Removal of a hydrolysate can restore the catalytic activity of an enzyme if it is suffering from product inhibition*. (2) *The hydrolase-catalyzed kinetic resolution would remain less competitive unless the unwanted enantiomer could be reused via racemization*.

Conclusions

As illustrated above, the following issues should be addressed when developing hydrolase-catalyzed resolution into an industrially viable process: (1) enantioselectivity, (2) product separation via partition, (3) volume efficiency, (4) product inhibition, and (5) reuse of the unwanted antipode. The last issue 5 is of such strategic importance that it should actually be the first to be contemplated.[32] Therefore, unless a chemical process could be developed that would allow the unwanted antipode to be reused via racemization or stereoinversion,[33] synthetic approaches other than kinetic resolution should be conceived. Proper chemical knowledge is also required to deal with product separation (issue 2). Specifically, enzymatic processes should be designed elaborately to allow separation between a left-over substrate and a digested product through partition.[34] In dealing with all of the other issues except the fourth issue, no methodology would be more rewarding than an intensive microbial screening as demonstrated by the case studies featuring the *K. oxytoca* hydrolase and *B. brevis* protease. As regards product inhibition (issue 4), chemical investigation into removing a causative hydrolysate from the reaction medium should also be effective along with efforts to identify a robust enzyme.

Received for review January 20, 2003.
OP034014B

(29) (a) For the reuse of (*S*)-**20a** via its oxidation to quinuclidin-3-one **23**, see ref 25. (b) Sternbach, L. H.; Kaiser, S. *J. Am. Chem. Soc.* **1952**, *74*, 2215. (c) For the synthesis of **23**, the immediate precursor to (±)-**20a**, see: Daeniker, H. U.; Grob, C. A. *Organic Syntheses*; John Wiley & Sons: New York, 1973; Collect. Vol. V, p 989.

(30) Ebbers, E. J.; Ariaans, G. J. A.; Houbiers, J. P. M.; Bruggink, A. Zwanenburg, B. *Tetrahedron* **1997**, *53*, 9417.

(31) (a) Nomoto, F.; Hirayama, Y.; Inoue, T. (Nagase & Co., Ltd.). Jpn. Tokkyo Kokai Koho 99 246,558, 1999. (b) For other application of Raney Co-catalyzed racemization, see: Fujii, A.; Fujima, Y.; Harada, H.; Ikunaka, M.; Inoue, T.; Kato, S.; Matsuyama, K. *Tetrahedron*: *Asymmetry* **2001**, *12*, 3235.

(32) For the comprehensive discussion on route selection, see: Ikunaka, M. *Chem.—Eur. J.* **2003**, *9*, 378.

(33) For the enatioconvergent chemoenzymatic processes, see: Hirohara, H.; Nishizawa, M. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1.

(34) (a) For the separation between an alcohol and an ester thereof via *O*-sulfation of the former, see: Yamano, T.; Kikumoto, F.; Yamamoto, S.; Miwa, K.; Mizuno, Y. *Chem. Lett.* **2000**, 448. (b) For the separation tactics employing hydrogen phthalate formation, see: Lloyd, R. C.; Smith, M. E. B.; Brick, D.; Taylor, S. J. C.; Chaplin, D. A.; McCague, R. *Org. Process Res. Dev.* **2002**, *6*, 762.