Organic Process Research & Development **2002**, *6*, 497−504

Selection, Purification, Characterisation, and Cloning of a Novel Heat-Stable Stereo-Specific Amidase from *Klebsiella oxytoca*, and Its Application in the Synthesis of Enantiomerically Pure (*R*)- and (*S*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic Acids and (*S*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionamide†

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Abstract:

We isolated, characterised, and cloned an enantio-specific amidase from *Klebsiella oxytoca* and used it to resolve (*R*,*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide, giving (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide. The (*S*)-amide could then be hydrolysed chemically to (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid. The process can therefore be adapted to produce both (*R*)- and (*S*)-enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid, or (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide. The biocatalytic step is part of a combined chemical and biocatalytic route that starts from ethyl trifluoroacetoacetate. The products typically have a purity of greater than 98% and ee values of essentially 100% after isolation. The process has been used to produce 100-g amounts of the (*S*)-acid, and successfully scaled up to produce 100-kg amounts of the (*R*)-acid, with the biotransformation carried out at the 1500-L scale.

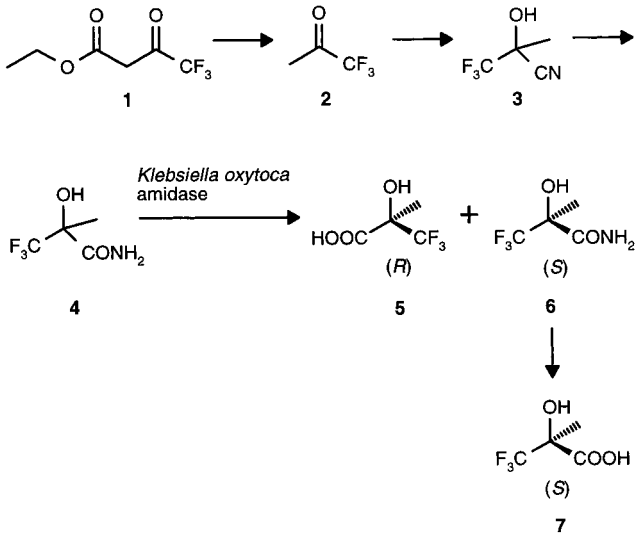

Figure 1. **Overall process for the manufacture of (*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid.**

Introduction

Biocatalysis is now an established method in the synthesis of organic compounds[1] and is especially useful for the production of chiral substances. At Lonza, chemists and biotechnologists work closely together to develop synthetic routes suitable for scale-up to kilogram and ton amounts.[2] Many of our target compounds are chiral intermediates for pharmaceuticals, and thus biocatalysis is often involved in the synthetic routes that we develop.

(*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid (**5** and **7**, respectively, Figure 1) are intermediates for the synthesis of a number of potential pharmaceuticals, which include ATP-sensitive potassium channel openers for the treatment of incontinence[3] and inhibitors of pyruvate dehydrogenase kinase for the treatment of diabetes.[4] When this work was started, the (*R*)- and (*S*)-acids had been synthesised by resolution of the (*R*,*S*)-acid with brucine,[5] and the (*S*)-acid had been synthesised with (*S*)-methylbenzylamine as the resolving agent.[3] Our aim was to develop efficient syntheses for these intermediates that yielded pure products with high ee values and that were suitable for large-scale production. The result was a process that combines chemical and biocatalytic steps. This report outlines the route chosen and describes the biocatalytic step in detail.

† Evelyne Schmid, Marie-Louise Hischier, Veronika Venetz, Josef Werlen, Patricia de Riedmatten, Jean-Paul Roduit, Bertin Zimmermann, and Roman Neumüller did not contribute to the discoveries described in WO98/01568, some of which are described in this paper.

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10.1021/op020025d CCC: $22.00
Published on Web 05/23/2002

Materials and Methods

Analytical Methods. Structures were routinely determined by NMR (Varian 400 MHz). The (*R,S*)-amide **4** was measured by GC. The sample (0.1 g) and pyradizine internal standard (0.1 g) were dissolved in 1.0 mL of acetonitrile, and 0.2 μL was injected. A HP5890 instrument equipped with a FID and a Optima-17 column (Macherey-Nagel no. 726022.25) were used. The inlet temperature was 180 °C, and the initial column temperature was 50 °C with a 10 °C/min gradient to 100 °C and a 25 °C/min gradient to 280 °C. The carrier gas was hydrogen, and the detector temperature was 320 °C. To monitor the biotransformation, the disappearance of the (*R*)-amide was followed by chiral GC analysis. Samples (0.5 mL) were filtered through a 0.2-μm membrane and mixed with 1 g of 1% butyramide in ethyl acetate and 0.8 g of NaCl. The samples were then shaken vigorously for 5 min, allowed to stand for 5 min and centrifuged, and the supernatant was analysed using a HP5890 instrument equipped with a FID and a FS-Lipodex E column (Macherey-Nagel no. 723368.25). The inlet temperature was 220 °C, and the initial column temperature was 125 °C, with a 4 °C/min gradient to 160 °C and a 25 °C/min gradient to 250 °C. The carrier gas was hydrogen, and the detector temperature was 300 °C. The (*R*)- and (*S*)-acid products were assayed by titration, and their ee values were measured using a chiral GC method. Samples (100 mg) were derivatised with a solution of 10% boron trifluoride in methanol (1 mL). The mixture was heated for 30 min at 70 °C and cooled, and hexane (1 mL) added and shaken. A saturated solution of NaCl (1 mL), was then added and shaken. The phases were allowed to separate, and 0.2 μL of the organic phase was injected onto GC. The GC and column (FS-Lipodex E) were the same as described above. The inlet temperature was 220 °C, and the initial column temperature was 60 °C, with a 5 °C/min gradient to 90 °C and a 20 °C/min gradient to 2000 °C. The carrier gas was hydrogen, and the detector temperature was 300 °C.

Ammonia was determined with a colorimetic method (Berthelot reaction). The solution to be tested (50 μL) was mixed with Solution A (2.5 mL) and Solution B (2.5 mL), and incubated at 30 °C for 30 min. The absorbance at 550 nm (A_{550}) was then read in a spectrophotometer, and the amount of ammonia present was determined by comparison with a standard curve prepared with known dilutions of an ammonium sulphate solution. Solution A consisted of 0.106 M phenol and 0.17 mM sodium nitroprusside. Solution B consisted of 11.0 mM sodium hypochlorite and 0.125 M sodium hydroxide. Amidase samples for LC−MS were diluted in 0.1% TFA and injected onto the system, which consisted of a Micromass Quatro II instrument with triple quadrupole analyser and a ABI 140B LC system. The column was a Vydac C_{18} (150 mm × 1 mm). The start solvent was 0.1% TFA, and the end solvent was 0.1% TFA in 90% acetonitrile. The column temperature was 40 °C, and the flow rate was 50 μL/min. A gradient from 20 to 100% of the end solvent was run over 45 min.

SDS-PAGE was carried out with the Phast System from Pharmacia (Amersham Biosciences) according to the instruction manual. Amino acid sequencing was carried out as described previously.[6]

Strain Enrichment and Screening. Bacterial strains were selected for their ability to use the (*R*)- or (*S*)-amide as the sole source of nitrogen for growth. For example, a soil sample (10 g) was put into 100 mL of 100 mM phosphate buffer, pH 7.0, allowed to stand for 10 min, and then filtered. The filtrate (5 mL) was then used as an inoculum in 25.0 mL of mineral salts medium[7] containing glycerine and (*R,S*)-amide in a C/N ratio of 5/1. The culture was incubated at 30 °C until microbial growth was observed. The culture was then serially transferred into fresh medium[7] with the racemic amide (0.41% w/v) as the sole nitrogen source plus glycerine (0.4% w/v) as the carbon source, to enrich microorganisms that grew with the amide as the nitrogen source. From the resulting mixed cultures, pure cultures of various microorganisms were obtained on agar media with the racemic amide as the sole nitrgen source. The stereospecificity of the individual pure cultures was then tested in liquid culture[7] with glycerine (0.4% w/v) as the carbon source and (*R,S*)-amide (0.41%) as the reaction substrate. Samples were taken at different stages of growth and tested by GC for the (*R*)- and (*S*)-amide, and the corresponding ee values were calculated. Alternatively the stereospecificity of the cells was tested in resting cell biotransformations. The cells were washed and re-suspended in 100 mM phosphate buffer, pH 7.0, with 0.5% (*R,S*)-amide. The mixture was incubated at 37 °C, and samples were taken for GC analysis at various times.

Generation of a Capsule-Negative Mutant of *K. oxytoca* PRS1. Mutagenesis was carried out as previously described,[8] using acridine ICR 191.

Enzyme Purification, Characterisation, and N-Terminal Amino Acid Sequence. During purification of the enzyme, the active fractions were detected by measuring the release of ammonia from the substrate with the colorimetric method described above. Subsequently the activity of the cell-free extract and pure enzyme was determined by the GC method. Cells of *K. oxytoca* PRS1 (200 mL; $OD_{650} = 21$ in 100 mM potassium phosphate buffer, pH 7.5) were broken open by passing them three times through a French press at 19 000 psi. Benzonase (1 μL/30 mL extract) was added and the extract centrifuged at 20000*g* for 15 min and at 100000*g* for 1 h. Benzonase is a genetically engineered endonuclease that degrades all forms of DNA and RNA. It is added to cell extracts to reduce the viscosity that results from the presence of DNA and which makes the enzyme purification process difficult. The supernatant (2.94 mg/mL protein) was heated for 10 min at 80 °C, and precipitated protein was removed by centrifugation. The supernatant (170 mL, 0.83 mg/mL) was loaded onto a HiLoad Q-Sepharose 26/10 chromatography column (Pharmacia) equilibrated with 50 mM potassium phosphate buffer, pH 7.5 (buffer A). Unbound

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protein was washed from the column with 130 mL of buffer A. A 500-mL gradient to 1 M NaCl in buffer A was run, flow rate 2.5 mL/min, and 5-mL fractions were collected and tested for activity. The most active fractions (30−37, 40 mL) were pooled and concentrated to 7.5 mL by ultrafiltration, and the buffer was exchanged to 10 mM potassium phosphate buffer, pH 7.5, by passing the sample through a gel-filtration column (Sephadex G-25 M, PD10, Pharmacia), and then loaded onto a hydroxyapatite column (5 mL, Bio-Scale CHTI, BioRad), also equilibrated with 10 mM potassium phosphate buffer, pH 7.5. A 90-mL gradient to 0.5 M potassium phosphate buffer, pH 7.5 was run, flow rate 2.0 mL/min, and 1.0 mL fractions were collected and tested for activity, which was seen in fractions 17−25 and 32−34. The protein, ($M_r \approx 37000$) in fraction 19 was judged to be pure by SDS-PAGE, as was that in fractions 33 and 34. The protein in fraction 20 was judged to be >95% pure. Fractions 20 to 25 were pooled, concentrated to 200 μL, and loaded onto a gel-filtration column (Superose 12, Pharmacia). Active fractions 23−26 were judged to be pure by SDS-PAGE. For protein sequencing the amidase was run on SDS-PAGE and Western blotted, and an N-terminal sequence was obtained from the band cut from the gel.[6]

For the amidase characterisation, a heat-treated cell-free extract was used. Cells of *K. oxytoca* PRS1/K16 (OD_{650} = 160) were broken open by passing three times through a French press at 19000 psi. Benzonase (1 μL/30 mL extract) was added and the extract centrifuged at 20000*g* for 1 h. The supernatant (typically 20 mg/mL protein) was heated for 10 min at 70 °C, and precipitated protein was removed by centrifugation. The supernatant (typically 2.0 mg/mL) was concentrated to approximately 5.0 mg/mL of protein and stored at −20 °C before use in the assays. The heating step therefore removed ~90% of unwanted protein.

The rate of reaction was directly proportional to the protein concentration, up to about 0.5 mg/mL. A concentration of 0.2 mg/mL was therefore routinely used in the assays. For the determination of the pH optimum of the enzyme the concentration of (*R*,*S*)-amide was 0.5% (32 mM), the temperature was 40 °C, and the following buffers were used: 100 mM MES, pH 6.5; 100 mM HEPES, pH 7.0 and 7.5; 50 mM phosphate, pH 8.0 and 8.5; 50 or 100 mM TRIS, pH 8.0 and 8.5; 50 or 100 mM borate, pH 9.0 and 9.5; 50 or 100 mM CAPS, pH 10.0, 10.5, and 11.0. The effect of temperature on the reaction was determined in 100 mM CAPS buffer, pH 10.0, with a substrate concentration of 0.5%. The effect of substrate concentration was determined at 60 °C, in 100 mM CAPS buffer. The K_m for the enzyme was calculated with the Enzfitter programme from BioSoft.

Isolation of the Chromosomal DNA from *K. oxytoca*. DNA from a fresh overnight culture from *K. oxytoca* PRS1/K17, grown in 100 mL of nutrient yeast broth at 30 °C, was isolated according to a modified method.[9] After centrifigation at 6500*g* and 4 °C for 15 min, the harvested cells were suspended in TRIS-HCl buffer (2.25 mL, 0.05 M, pH 8.0, 10% (w/v) sucrose). Lysozyme solution (0.375 mL, 10 mg/mL, 0.25 M TRIS-HCl buffer, pH 8.0) and 0.9 mL of aqueous EDTA solution (0.1 M, pH 8.0) were added to the suspension followed by incubation on ice for 10 min. After the addition of 0.45 mL 5% (w/v) SDS and 0.05 mL of ribonuclease (10 mg/mL in H_2O), the suspension was incubated at 37 °C for 30 min. The incubation was continued for another 2 h after the addition of 0.4 mL of Pronase (20 mg/mL in H_2O) and a small amount (spatula end) of proteinase K. The suspension was mixed with 4.3 g of CsCl, centrifuged at 40000*g* for 30 min at 20 °C, and 0.25 mL of ethidiumbromide (10 mg/mL in H_2O) was added. The mixture was centrifuged at 246000*g* at 20 °C for 8 h (Vti 65.2 rotor). The DNA band was isolated from the CsCl gradient and diluted with TRIS-EDTA buffer (10 mM TRIS-HCl, pH 8.0, 1.0 mM EDTA). Ethidium bromide was extracted three times with *n*-butanol (H_2O saturated). After precipitation with 2-propanol, the solution of DNA in TRIS-EDTA buffer was incubated for 15 min at 65 °C and stored at 4 °C.

Restriction and Ligation of Chromosomal DNA and Plasmids. DNA from *K. oxytoca* PRS1/K17 (0.005 mg) and vector DNA (pBluescript KS+, 0.0045 mg) were each digested with 20 U *Hin*dIII in 0.1 mL of restriction buffer at 37 °C for 6.5 h. The DNA was precipitated with ethanol, dried in a Speed Vac concentrator, and diluted with 0.05 mL of ligation buffer (20 mM TRIS-HCl, pH 7.2, 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.6 M ATP). The samples were pooled and after the addition of T4-DNA (1 unit ligase/per μg DNA) ligated overnight at 13 °C. After the precipitation of the DNA from the ligation mixture with 2-propanol, it was suspended in water (0.03 mL) and used directly for the transformation of the *Escherichia coli* recipients.

Transformation and Selection of *E. coli* Cells. Competent cells of *E. coli* XL1-Blue MRF′ were electrotransformed with the overnight ligation mixture.[10] For the selection of the plasmid the nutrient agar contained ampicillin (0.1 mg/mL). Screening for positive insert ligations was carried out on medium containing isopropyl-β-D-thiogalactoside (IPTG, 0.5 mM) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 0.03 mg/mL) at 37 °C. Nearly all clones contained a *Hin*dIII insert. The transformation frequency was 1.7×10^8 colony-forming units/mL.

Screening of the *Hin*dIII Clones for the (*R*)-Specific Amidase. The *E. coli* transformants containing a *Hin*dIII hybrid plasmid were plated on mineral salts medium[7] with glycerol (0.4% v/v) as carbon source, (*R*,*S*)-amide (0.2% w/v) as the sole nitrogen source, and ampicillin (0.05 mg/mL) for plasmid stability. Only clones containing the intact amidase gene (*sad*) on the DNA insertion within the plasmid were able to grow on this medium. All selected clones contained a hybrid plasmid of the same *Hin*dIII insertion fragment with a size of 2.73 kb. This plasmid was characterised physically.

Localisation of the Gene Coding for the Amidase (*sad*) on the Cloned *Hin*dIII Fragment. Restriction analysis with the enzymes *Xho*I, *Dra*II, *Sma*I, *Pst*I, *Sal*I, and *Bam*HI was

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carried out,[11] and a preliminary restriction map was constructed. Mixed DNA oligomers corresponding to the N-terminal peptide sequence of the amidase were synthesised. The DNA from the plasmid was digested with various restriction enzymes (*Dra*II, *Sma*I, *Eco*RI, *Hin*dIII, and *Bam*HI), and fragments were separated by agarose gel electrophoresis (0.6%, w/v) and then analysed by Southern blot hybridisation. The probe of mixed oligomers was 3′-end-labeled with digoxygenin, and the hybridisation and detection were carried out as described previously.[11] The hybridisation of the oligonucleotide mixture representing the N-terminal peptide sequence allowed the identification of a 1.52-kb *Dra*II−*Bam*HI restriction fragment on the hybrid plasmid, which was then sub-cloned into pBluescript KS+ under the control of the strong P_{lac} promotor to improve its expression.

Biotransformations. Biotransformation reactions were carried out at scales from 1.0 mL to 1500 L. For small-scale reactions, the pH was controlled by the presence of buffer. On larger scales the pH was controlled automatically by the addition of NaOH.

Synthesis of (*R*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic Acid at 100-kg Scale: (i) Ethyl Trifluoroacetoacetate to (*R,S*)-Amide.[5,12] *(a) Ethyl Trifluoroacetoacetate **1** to Trifluoroacetone **2**.* Concentrated sulphuric acid was added slowly to water in a glass-lined reactor (10−95 °C) followed by ethyl trifluoroacetoacetate at 90−102 °C. When the reaction was complete, the ethanol produced in the reaction was captured in a condenser and returned to the reactor, whereas the product, trifluoroacetone, was captured by a second condenser positioned in series with the first, and collected in a cooled container.

*(b) Trifluoroacetone to Cyanohydrin **3**.* Trifluoroacetone was added slowly to a solution of NaCN (25%) at <10 °C in a glass-lined reactor. Concentrated HCl was added to the resulting white suspension at <20 °C until the pH was 1.0. At this pH a two-phase solution formed. The lower phase, containing the cyanohydrin, was separated and used for the next step.

*(c) Cyanohydrin **3** to (R,S)-Amide **4**.* The cyanohydrin **3** was added slowly to concentrated sulphuric acid at 80 °C in a glass-lined reactor. When the reaction was complete, water was added slowly, the mixture was cooled to 0 °C, and the precipitated (*R,S*)-amide was collected by centrifugation.

Synthesis of (*R*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic Acid at 100-kg Scale: (ii) Biotransformation. The biotransformation was carried out in a volume of 1500 L. The substrate concentration was 10% (w/v), the temperature was 37 °C, and the pH of 8.0 was maintained by the addition of NaOH. The reaction was started by the addition of washed whole cells of *E. coli* containing the recombinant amidase. The cells for the biotransformation were grown to an OD_{650} of about 8 in a separate fermenter, washed in acetate buffer, and concentrated to a OD_{650} of 100 in a microfiltration unit. Stabilisation of the amidase in the biomass was achieved by heating to 70 °C for 10 min.

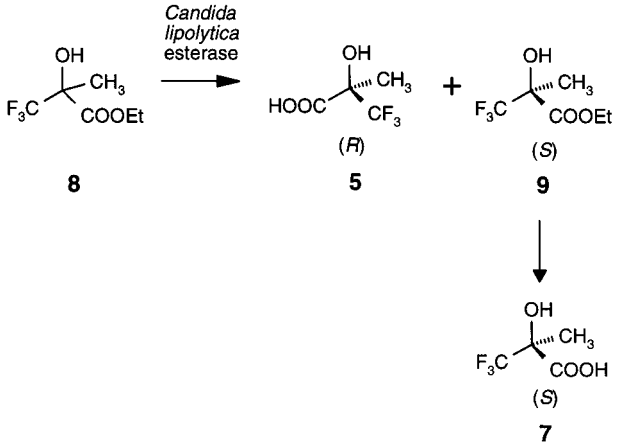

Figure 2. **Resolution of (*R,S*)-ethyl-3,3,3-trifluoro-2-hydroxy-2-methylpropionate with the esterase from *Candida lipolytica*.**

Typically the biotransformation was carried out with the cells at OD_{650} = 1.0−5.0. The biotransformation was stirred gently, monitored by gas chromatography of the substrate, and stopped by adjusting the pH to 4.0 with phosphoric acid. The cells were then removed by microfiltration, and the product solution was passed through a 70-kDa ultrafiltration unit to remove any protein from lysed cells. The product solution was then concentrated to about 12% (w/v) with respect to the (*R*)-acid, adjusted to pH 7.0, and delivered to the chemistry pilot plant for isolation of the (*R*)-acid.

Synthesis of (*R*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic Acid at 100-kg Scale. (iii) Product Isolation.[5] The product solution from the biotransformation was extracted three times with ethyl acetate to remove the (*S*)-amide. The aqueous phase was then adjusted to pH 1.0 with HCl and the product extracted three times with a methyl *tert*-butyl ether/toluene mixture. Methyl *tert*-butyl ether was then removed from the pooled organic extracts by vacuum distillation. The precipitated product was collected by centrifugation and dried at 30 °C and <50 mbar. The drying conditions must be closely controlled to avoid sublimation of the product.

Results and Discussion

Choice of Synthetic Route and Strain Selection. We evaluated and tested a number of possible routes, some of which were purely chemical, and others that involved one or more biocatalytic steps. The biocatalytic routes were the most promising. For example the resolution of (*R,S*)-ethyl-3,3,3-trifluoro-2-hydroxy-2-methylpropionate **8** with the esterase from *Candida lipolytica* resulted in the (*S*)-ester **9** with an ee value of 99%[13] (Figure 2). However, this route was not developed further because of the amount and resulting cost of the enzyme required to complete the reaction in a reasonable time (another biocatalytic route to the (*R*)-acid involving the lipase-catalysed resolution of esters of the cyanohydrin **3** was reported after we had completed this work).[14] Other possible routes with one or more biocatalytic

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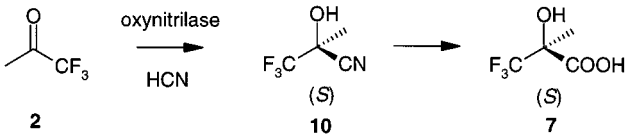

Figure 3. **Possible route to 3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid using an oxynitrilase. According to the choice of enzyme, it could be possible to form either the (*R*)- or the (*S*)-enantiomer. The route to the (*S*)-cyanohydrin 10 and (*S*)-acid 7 is shown.**

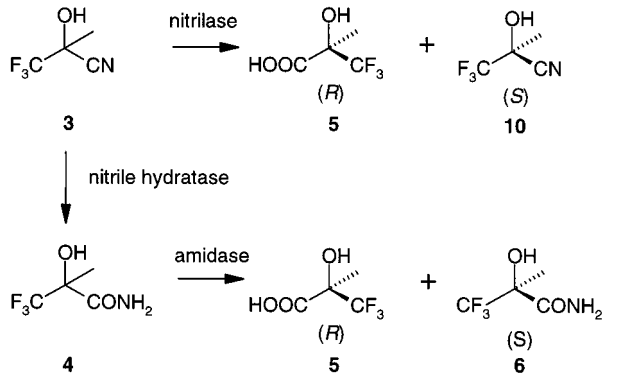

Figure 4. **Possible routes starting from the racemic cyanohydrin 3. Nitrilases convert nitriles to the corresponding acids and are sometimes stereospecific. Nitrile hydratases convert nitriles to amides and are also sometimes stereospecific. Amidases convert amides to the corresponding acids and are often stereospecific. The routes to the (*R*)-acid are shown. The corresponding routes to the (*S*)-acid could also be possible.**

steps included those involving an enantioselective oxynitrilase reaction (Figure 3) and various routes starting from the racemic cyanohydrin **3** (Figure 4). Screening for enantioselective oxynitrilases[15] and for enantiospecific nitrilases[16] was started but discontinued when the amidase route (below) was found to be successful.

The route chosen for development and scale-up is shown in Figure 1.[17] It starts with ethyl trifluoroacetoacetate **1**, which is converted chemically in three stages to the racemic amide **4**. Bacterial strains that grew with the (*R*,*S*)-amide as the sole source of nitrogen were screened for enantiospecificity by chiral GC analysis of the reaction substrate. In this way a number strains were identified that could hydrolyse either the (*S*)- or the (*R*)-amide to the corresponding acid with a high degree of specificity, and at a rate suitable for an industrial biotransformation. The most promising strain, PRS1, was identified as *K. oxytoca* and was chosen for process development. It contained an amidase specific for the (*R*)-amide.

The biotransformation with the (*R*)-specific amidase fom *K. oxytoca* resolves the (*R*,*S*)-amide **4**, giving the (*R*)-acid **5**. The (*S*)-amide **6** remains unconverted. The (*R*)-acid and (*S*)-amide are then separated by extraction. Both can then be isolated as products, or the (*S*)-amide can be hydrolysed

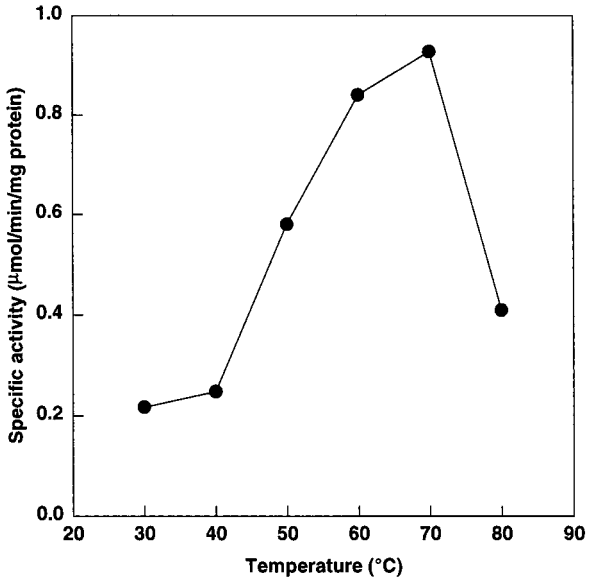

Figure 5. **Effect of temperature on the hydrolysis of the (*R*)-amide by the amidase from *K. oxytoca*.**

chemically to the (*S*)-acid **7**, which can then be also isolated as a product. A nitrile hydratase (Figure 4) was found that converted the racemic cyanohydrin **3** to the racemic amide **4**, but this biotransformation was not used because it had no great advantage over the corresponding chemical reaction.

The final choice of route was decided by a number of factors: (i) ethyl trifluoroacetoacetate is a Lonza product, making backward integration of the process easy; (ii) Lonza has expertise in large-scale cyanide chemistry, which was used for the synthesis of the substrate for the biotransformation, and; (iii) the successful enrichment and isolation of a suitable biocatalyst that yielded products of high purity and with high ee values.

Mutagenesis of the Wild-Type *K. oxytoca* Strain PRS1. The handling of microorganisms and downstream processing are important considerations in the design and operation of any process that involves biotransformation. Although the wild-type *K. oxytoca* strain PRS1 performed well in the biotransformation, it had a slime capsule that is characteristic of many representatives of the genus *Klebsiella*, and that made product purification difficult. Using chemical mutagenesis, capsule-negative strains PRS1/K16 and PRS1/K17 were obtained that could still carry out the biotransformation but that were more suitable for handling and product purification on large scale than the wild-type strain PRS1.

Amidase Purification and Characterisation. The amidase was heat-stable, with maximum activity at 70 °C (Figure 5). This heat stability facilitated purification of the enzyme. Heating the cell-free extract to 80 °C for 10 min and removing precipitated protein by centrifugation resulted in a 5-fold increase in the specific activity of the enzyme preparation. The amidase was then purified from the heat-treated solution by chromatography on anion-exchange, hydroxyapatite, and gel-filtration columns (Table 1). The purified enzyme had a subunit $M_r \approx 37\,000$ by SDS-PAGE analysis (Figure 6). LC−MS showed a peak at M_r 36347, which confirmed the SDS-PAGE estimate. The amidase had a pH optimum of about 9−10 (Figure 7) and had Michaelis−

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(15) Effenburger, F.; Hörsch, B.; Weingart, F.; Ziegler, T.; Kühner S. *Tetrahedron Lett.* **1991**, 2605−2608.
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Table 1. **Purification of the amidase from *K. oxytoca* PRS1**

step	volume (mL)	protein (mg)	activity[a] (μmol/min)	specific activity (μmol/min/mg protein)	purification (fold)	recovery (%)
cell-free extract	250	735	42.3	0.058	0	100
80 °C heat treatment	170	141	42.3	0.3	5.2	100
anion exchange	40	16				
hydroxylapatite	7	2.36				
gel-filtration	5.0	1.0	2.7	2.7	46.6	6.4

[a] pH 10, 40 °C, 1.0% (*R,S*)-amide as substrate.

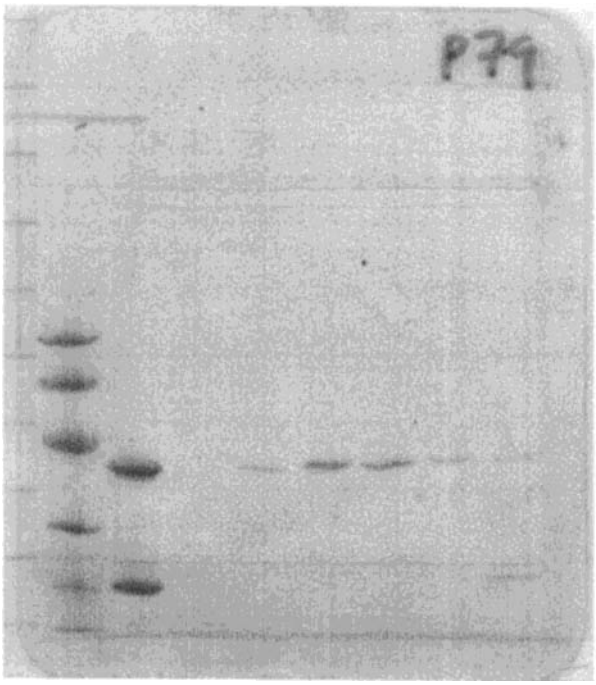

Figure 6. **SDS-PAGE analysis (Coomasie Blue stained 10−15% gel, Phast System; Amersham Pharmacia Biotech) of the purified amidase from *K. oxytoca.* The gel shows: lane 1 (far left), molecular weight markers (14.4, 20.1, 30.0, 45.0, 66.0, and 97.0 kDa); lane 2, the material loaded on to the final gel-fitration chromatography column; lanes 3−8, fractions 22−27 from the gel-filtration column.**

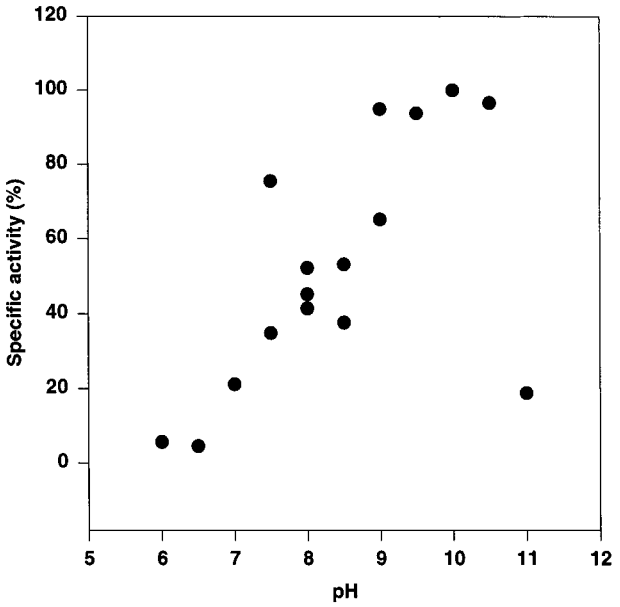

Figure 7. **Effect of pH on the hydrolysis of the (*R*)-amide by the amidase from *K. oxytoca*.**

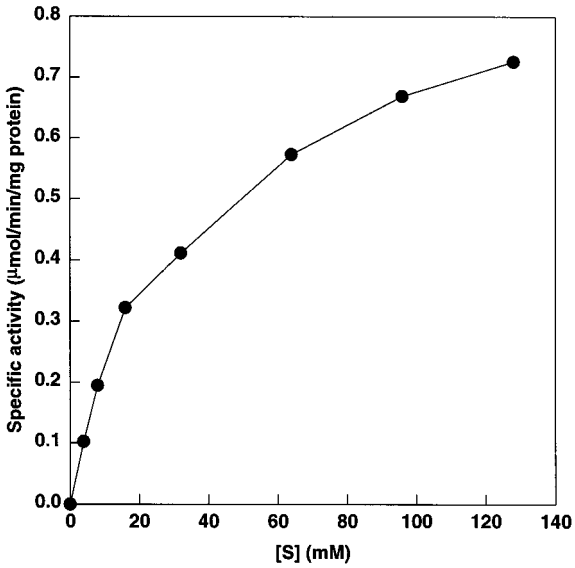

Figure 8. **Effect of substrate concentration on the hydrolysis of the (*R*)-amide by the amidase from *K. oxytoca*. The substrate concentration is that of the (*R*)-amide.**

Menten-type kinetics, with a K_m value of 32 mM (Figure 8), a catalytic constant or catalytic-centre activity value (k_{cat} or k_0) of 1933/s and a specificity constant (k_{cat}/K_m) of 6.0×10^4/M/s.[18] The N-terminal amino acid sequence of the amidase was: M.K.W.L.E.E.S.I.M.A.K.R.G.V.G.A.S.R.K.P.

Generation of a Recombinant Production Strain. A further step was to clone the amidase gene into *E. coli*. The reasons for doing this were: (i) to improve safety; *K. oxytoca* is a risk class 2 microorganism. Transfer of the amidase gene to a GRAS host such as an *E. coli* K12 derivative facilitated handling and official registration procedures for the production process, (ii) the productivity of the biotransformation was improved by cloning the amidase gene under the control of a strong promoter to improve its expression, (iii) to avoid the slime-capsule problem, and (iv) to have the possibility to use other microorganisms with, for example, higher substrate or product tolerances, as hosts for the cloned gene.

The amidase gene was isolated and inserted into a vector suitable for expression in *E. coli* using standard molecular biological techniques. Chromosomal DNA was isolated from a capsule-negative mutant of *K. oxytoca*, digested with various restriction enzymes and ligated into a screening

(18) Cornish-Bowden, A. *Fundamentals of Enzyme Kinetics*; Portland Press: London, 1995.

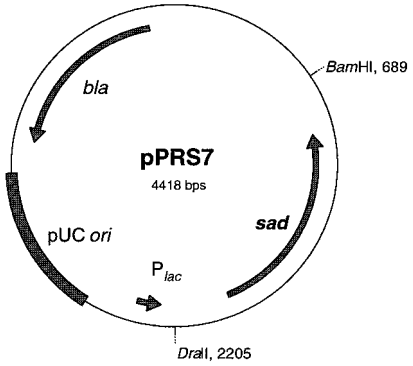

Figure 9. **Plasmid pPRS7, with the *K. oxytoca* amidase gene (*sad*).**

vector. Cells with plasmids containing *K. oxytoca* DNA were selected for the presence of the amidase on growth medium containing the racemic amide as the sole source of nitrogen. Only those cells containing the cloned amidase could hydrolyse the amide, release ammonia, and grow. All of the selected clones contained a plasmid with the same *Hin*dIII insertion fragment of 2.73 kb. The amidase gene was localised with a mixed oligomer corresponding to a portion of the N-terminal amino acid sequence of the amidase. Although the cloning of the amidase gene from *K. oxytoca* into *E. coli* led to measurable amidase activity in the host, the activity was no higher than in the *Klebsiella* strain. This was because expression of the amidase gene was still under the control of the natural promoter. Subcloning experiments were therefore carried out to place the gene behind the powerful P_{lac} promoter (IPTG inducible) present on the cloning vector. The resulting clone (pPRS7, Figure 9) had >10 times more amidase activity (1.7 g/L/h/OD_{650} = 1.0) than the original wild-type strain, *K. oxytoca* PRS1 (0.11 g/L/h/OD_{650} = 1.0).

Biotransformations and Scale-Up. The heat-stable amidase from *K. oxytoca* is robust and stable and does not require cofactors. During the research and development phases of the project, biotransformations were carried out with the wild-type *K. oxytoca* PRS1, with the capsule-negative mutants of *K. oxytoca*, and with the recombinant *E. coli* strains. Biotransformations were carried out with washed whole cells, with cells that had been heated to 70 °C for 10 min, and with cell-free extracts that had been heated to 70 °C for 10 min. The heat treatment at 70 °C stabilises the amidase activity in whole cells and cell-free extracts, presumably by inactivating proteases. It also leads to purification of the enzyme in cell-free extracts by precipitating unwanted protein. A process with a pure enzyme would simplify DSP, since large amounts of contaminating proteins and other cellular constituents would not be introduced in to the biotransformation.

The biotransformation was investigated with various substrate concentrations and with various amounts of biocatalyst at various pH values, temperatures, and in volumes from 1 mL to 1500 L. The biotransformation can be used to produce enantiomerically pure (*R*)-acid, (*S*)-acid, or (*S*)-amide. We selected conditions for scale-up so that the biotransformation reaction would reach completion, that is complete hydrolysis of the (*R*)-amide, with virtually no hydrolysis of the (*S*)-amide, to obtain the highest possible yield. However, if the amount of biocatalyst is too high, there is a danger that the reaction will over-run and the undesired enantiomer, the (*S*)-amide, will also be hydrolysed by the enzyme. This is not a problem if the desired product is the (*S*)-acid or the (*S*)-amide (because any (*R*)-/(*S*)-acid mixture formed in the biotransformation is separated from the (*S*)-amide, which can then be isolated or converted to pure (*S*)-acid), but is important if the desired product is the pure (*R*)-acid (because any (*S*)-acid formed in the biotransformation cannot be separated from the (*R*)-acid).

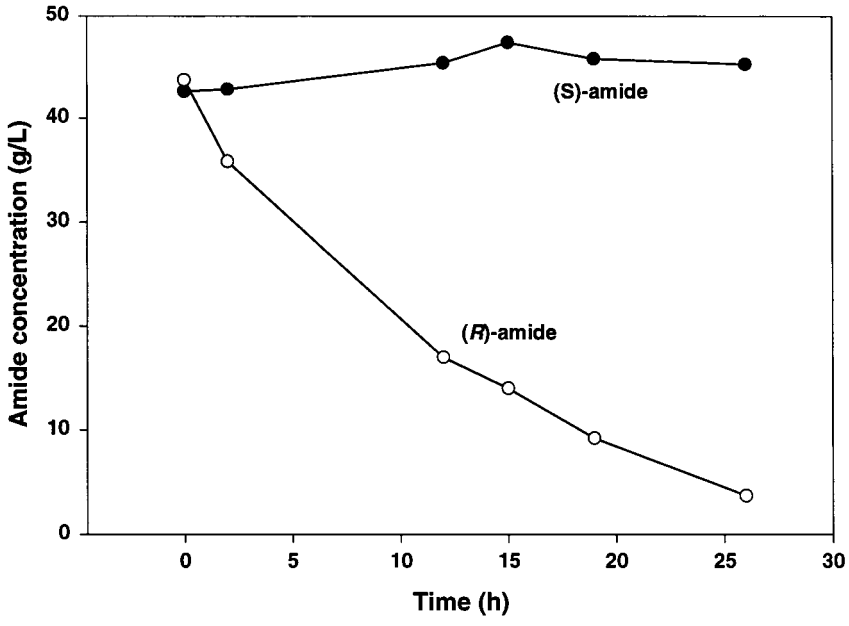

Figure 10. **Biotransformation at 1500-L scale. The reaction was monitored by chiral GC analysis of the (*R*)- and (*S*)-amides. The (*R*,*S*)-amide start concentration was 100 g/L, the temperature was 37 °C, the pH was maintained at 8.0, and heated-treated recombinant cells of *E. coli* with the *K. oxytoca* amidase were added to give an OD_{650} = 5.**

The process was run to produce the (*S*)-acid at 100-g scale and to produce the (*R*)-acid at 100-kg scale, where the biotransformation was carried out in a volume of 1500 L (Figure 10). The biotransformation remained virtually identical during scale-up from 1 to 1500 L, and our detailed knowledge of the enzyme made scaling-up easier. The step- and overall yields for the process at 100-kg scale were good, with the biotransformation yield almost reaching the theoretical maximum of 50%. The purity of the isolated (*R*)-acid was >98%, and the ee value was essentially 100%.

For the whole process attention was paid to safety, efficiency, and economics. The biotransformation was carried out in aqueous solution (which is often the case with biotransformations). Large volumes of aqueous solution containing relatively low product concentrations are difficult to handle in chemical plants (compared with "normal" processes in organic solutions); therefore, we paid particular attention to the delivery of a biotransformation product solution suitable for downstream processing. It was subjected to ultrafiltration through a 70-kDa membrane to remove proteins that could cause foaming during extraction, and contamination of the product. Concentration by thin-film evaporation then removed as much water as possible before transfer to the chemical pilot plant.

Summary

We have developed processes for the manufacture of enantiomerically pure (*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid, and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide. The (*S*)-acid has been produced at the 100-g scale, and we have scaled up the process for the (*R*)-acid to 100-kg amounts. The biotransformation step was run at 1500 L, using a recombinant strain of *E. coli* that contains a heat-stable amidase from *K. oxytoca*.

We are currently working on further improving the economics of the biotransformation, by cloning the amidase gene into strains of *E. coli* that are suitable for high cell density fermentation, and by investigating various forms of the biocatalyst: whole cells, heated cell mass, immobilised whole cells, and free, immobilised, and encapsulated enzyme.

GLOSSARY

M_r	relative molecular mass
OD	optical density
K_m	Michaelis constant
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
g	centrifugal force
LC−MS	liquid chromatography−mass spectrometry
GRAS	*g*enerally *r*egarded *a*s safe
MES	2-(*N*-morpholino)ethanesulfonic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid
TRIS	tris-(hydroxymethyl)methylglycine
CAPS	3-(cyclohexylamino)propanesulfonic acid
kb	kilobases

Acknowledgment

We thank Luc-Alain Savoy (M-Scan SA) for LC−MS, Séverine Frutiger-Hughes and Graham Hughes (CMU, Geneva) for amino acid sequencing, Miranda Anthenien, Georgette Hosennen, Judith Mazotti, Georges Kalbermatten and Klaus Heinzmann for experimental work, Alain Heinzmann, Michael Carlen and Hans-Jörg Heynen for running the biotransformation at pilot scale, and Candid Stoffel and Franz Williner for running the chemical steps at pilot scale.

Received for review February 10, 2002.

OP020025D