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Large Scale Preparation of Chiral Building Blocks for the P_3 Site of Renin Inhibitors

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Abstract—Racemic ethyl 2-benzyl-3-(*tert*-butylsulfonyl)propionate (**1**) and racemic ethyl 2-benzyl-3-[[1-methyl-1-((morpholin-4-yl)carbonyl)ethyl]sulfonyl]propionate (**3**) were enantioselectively hydrolyzed by subtilisin Carlsberg generating the respective (*S*)-acids used as building blocks for renin inhibitors. The esters were readily converted as emulsions at elevated temperature, in a suspended form or a two-phase-liquid system. The enzyme maintained its excellent selectivity and a good activity also at high initial substrate concentrations (up to 50 % w/w). The enzymatic reaction and work-up were optimized and scaled up. Emulsion problems during work-up encountered with these highly concentrated mixtures were solved by application of a disk separator for phase separation.

Introduction

The renin-angiotensin system (RAS) is a multiregulated proteolytic cascade that produces the potent pressor and aldosteronogenic peptide angiotensin II (Ang II). In a first step the aspartic proteinase renin selectively cleaves its protein substrate angiotensinogen to release the decapeptide angiotensin I, which in turn is processed by angiotensin converting enzyme to the octapeptide Ang II. The blockade of the initial step in the enzymatic cascade of the RAS is a promising concept for the treatment of hypertension.

In the last decade, intensive work worldwide on the design of renin inhibitors has yielded several classes of potent compounds. Most of these inhibitors are transition-state mimetics of the P_1–P_1' scissile bond (Figure 1) combined with amino acid residues or analogues thereof.[1] Much work has been focused on the minimization of the peptide character of renin inhibitors to overcome the drawbacks of substrate-analogous peptides such as instability towards enzymatic cleavage, biliary excretion and low oral bioavailability. In the course of our studies on renin inhibitors we synthesized a series of mimetics incorporating the P_4–P_3 binding elements.[2,3]

Various methods for the preparation of enantiomerically pure P_4–P_3 mimetics have been published. Among these are the classical co-crystallization with chiral amines,[4,5] the derivatization as diastereoisomeric amides,[6–8] the synthesis of chiral intermediates following Evans's methodology,[8–10] using lipase-catalyzed reactions[11] or the asymmetric hydrogenation of unsaturated derivatives.[5,12]

A classical chemoenzymatic approach for the kinetic resolution of racemic N-protected phenylalanines and

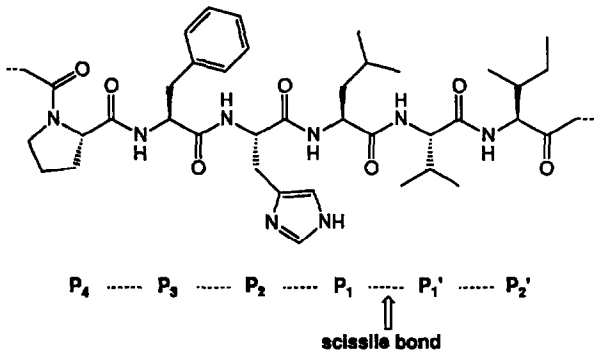

Figure 1. Substrate side-chain positions (P4–P_2') for angiotensinogen are illustrated using the notation of Schechter and Berger.

analogues thereof is based on the enantioselective hydrolysis of their esters by means of serine proteases, namely α-chymotrypsin or subtilisins BPN' and Carlsberg generating the corresponding L-acids.[13–22] The same configurational selectivity has been observed also for 2-benzyl succinate esters with α-chymotrypsin.[23] For two of our renin inhibitors, remikiren and ciprokiren (Figure 2), we decided to produce the chiral phenylalanine analogue building blocks **2** and **4** with the aforementioned protease approach employing the corresponding ethyl esters **1** and **3** as substrates. Just prior to the end of our work, the resolution of a series of 2-benzyl-3-sulfonamidopropionic acid esters similar to **3** was reported[18,24] using subtilisin Carlsberg. Alternatively, 2-(1-naphthylmethyl)-3-morpholinocarbonyl propionic acid methyl ester, a compound related to **3**, has recently been resolved using *Bacillus brevis* IAM1031 or pancreatin.[25]

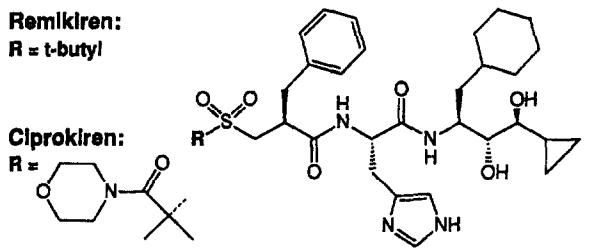

Figure 2. Renin inhibitors remikiren and ciprokiren.

Since remikiren as well as ciprokiren were both selected for clinical development, bulk quantities of the (*S*)-acids **2** and **4** had to be prepared. The precursor racemic ethyl 2-benzyl-3-(*tert*-butyl-sulfonyl)propionate **1** was prepared according to a modified published procedure.[7] Racemic ethyl 2-benzyl-3-[[1-methyl-1-((morpholin-4-yl) carbonyl) ethyl] sulfonyl]-propionate (**3**) was synthesized using a similar procedure published by Branca *et al.*[3] The following paper describes the efficient large scale preparation of the two (*S*)-acids **2** and **4** by the use of extremely cheap detergent proteases.[26]

Results

Preliminary experiments to produce gram-amounts of building block **2** and **4** were carried out successfully with α-chymotrypsin at 27–30 °C in the presence of around 2 % EtOH as cosolvent (*c.f.* reference 27). The acids of (*S*)-configuration were obtained in 99 % *e.e.* at nearly 50 % conversion (confirmed by X-ray analysis of a derivative, results not shown). In order to be capable of treating larger amounts of substrate we looked for a cheaper enzyme source. An enzyme screening with ester **1** employing cheap commercial bulk proteases revealed several enzymes with excellent enantioselectivity (E > 300),[28] namely Alcalase®2.0 T, Optimase®M-440, Savinase®6.0 T (from *Bacillus* sp.), HT Proteolytic®200 (*Bacillus subtilis* var.) and Prozyme®6 (*Aspergillus* sp.). The most active and at the same time cheapest enzyme preparations were Alcalase® and Optimase®, both fermentation products of *Bacillus licheniformis*. Various enzyme preparations from *Bacillus licheniformis* are available as detergent grade granules (Alcalase®2.0 T from Novo Nordisk; Optimase®M 440 from Solvay Enzymes, formerly Miles Kali-Chemie) and liquid (Alcalase®2.5 L from Novo Nordisk), respectively, or as food grade water-miscible liquid (Protease®L 660 from Solvay Enzymes); the major component of all is subtilisin A, better referred to as subtilisin Carlsberg, an endoproteinase of the serine type. It catalyses the hydrolysis of proteins and amino acid esters[29] and is stable in the presence of various organic solvents in concentrations up to 30 %.[18,21] It has been widely used as a detergent additive.

In order to get best hydrolysis conditions for the large scale production of the remikiren building block **2**, optimization of several reaction parameters was carried out arbitrarily using Optimase® M 440. The goal was to minimize reaction volume and to increase the hydrolysis rate while keeping the chemical purity and enantiomeric excess of **2** above 99 % and work-up simple. Reduction of the reaction volume is crucial for lowering production costs and can be achieved either by increasing the initial concentration of starting material in the reaction medium or by increasing the concentration of the sodium hydroxide solution used for maintaining the pH during hydrolysis. Increasing the substrate concentration made it necessary to also increase the enzyme concentration if the reaction time was not to exceed 2 days (Table 1). However, even under completely non-physiological conditions, at a substrate to buffer ratio of 1:1 (w/v), the enzyme still shows satisfactory activity, providing a product with still excellent chemical and enantiomeric purity (both >99 %). As at higher substrate concentrations work-up became more and more tedious due to emulsion problems, an initial concentration of 25–50 g **1** in 100 mL buffer seemed to be optimal. Very high titrating agent concentration (9 M NaOH) clearly affected hydrolysis rate, especially at high substrate concentrations (Table 2), possibly due to exposition of the enzyme to excessive salt concentration and/or product inhibition. Titrating agent concentrations of 2–4 M NaOH were acceptable (results not shown). Increasing NaOH concentration had a much more pronounced effect than increasing the substrate concentration. Thus, in order to have a high time space yield it is better to work with a high substrate concentration and titrate with a moderately concentrated sodium hydroxide solution than *vice versa*. Moreover, work-up becomes more tedious with increasing NaOH concentration as the emulsions encountered were more stable. Variation of the pH (Table 3) demonstrated the expected acceleration of the hydrolysis rate with increasing pH. Whereas at values up to pH 9.0 acid **2** was yielded in excellent quality, at pH 10 a noticeable rate of unspecific hydrolysis occurred. Increasing the reaction temperature revealed a slight decrease of activity at 45 °C (Table 4) probably owing to minor denaturation or

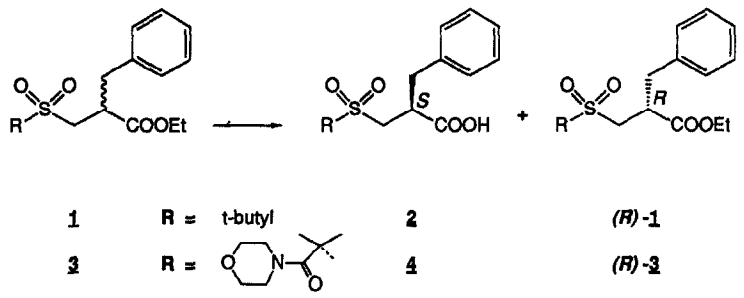

Figure 3. Enantioselective hydrolysis of racemic 2-benzyl-3-sulfopropionyl esters **1** and **3** by subtilisin Carlsberg.

degradation of the enzyme. Due to substrate precipitation at lower temperatures (mp of **1** is 36–39 °C) it is necessary to keep the temperature at a minimum of 37 °C, thus limiting the useful temperature range to 38–43 °C. In search for the optimal enzyme formulation the different commercial bulk preparations of subtilisin Carlsberg–Alcalase®, Optimase® and Protease® — have been tested using our initial standard conditions (Table 5). All of them yielded very good results with respect to chemical purity and enantiomeric excess (>99 % each), but Solvay Protease® L 660 had a much higher activity than the others based on weight.

Table 1. Performance of Optimase® M 440 at various substrate and enzyme concentrations[a]

amount of **1** (g)	amount of Optimase (g)	time (h) elapsed for 46 % conversion	g of **1** converted per g enz · h
6.3	0.4	13	0.56
12.6	0.8	22	0.33
25.2	1.6	34	0.21
50.4	4.0	45	0.13
100.0	10.0	53	0.09

a:Reaction conditions: Ester **1** was emulsified at 39–40 °C under vigorous stirring in 100 mL of 0.1 M sodium phosphate buffer, pH 7.5. After addition of the enzyme the pH was maintained by the controlled addition of 1 N NaOH.

Table 2. Performance of Optimase® M 440 at different substrate and titrating agent concentrations[a]

amount of **1** (g)	amount of enzyme (g)	[NaOH] (M)	time (h) elapsed for 30 % conversion	g **1** converted per g enzyme · h
12.6	0.8	1	7	0.68
12.6	0.8	9	10	0.47
100	10	1	15	0.20
100	8	9	69	0.05

a:For reaction conditions see Table 1.

Table 3. Performance of Optimase® M 440 at different pH's[a]

pH	time (h) elapsed for 47 % conversion	g of **1** converted per g enzyme · h
7.5	24	0.31
8.0	19	0.39
8.5	16	0.46
9.0	15	0.49

a:Reaction conditions: 12.6 g of **1** and 0.8 g of enzyme were employed under the conditions of Table 1.

Table 4. Performance of Optimase® M 440 at different temperatures[a]

temperature °C	time (h) elapsed for 33 % conversion	g of **1** converted per g enzyme · h
30-31	16	0.32[b]
39-40	5	1.04
44-45	5	1.04[c]

a:Reaction conditions: 6.3 g of **1** and 0.8 g of enzyme were employed under the conditions of Table 1. b: The starting material precipitated. c:Slowed down more rapidly on further progress of hydrolysis than reaction at 39–40 °C.

Table 5. Performance of various preparations of Optimase® and Alcalase®[a]

Enzyme preparation (form)	time (h) elapsed for 40 % conversion	g of **1** converted per g enzyme · h
Optimase M 440 (solid)	14	0.46
Protease L 660 (liquid)	11	0.58
Alcalase 2.0 T (solid)	16	0.40
Alcalase 2.5 L (liquid)	14	0.46

a:12.8 g of **1** and 0.8 g or mL of enzyme were applied as in Table 1.

Based on the above parameter screening the following optimized procedure was elucidated: 25–50 g of **1** emulsified in 100 mL of buffer pH 8.5 by vigorous stirring was hydrolyzed with Solvay Protease® L 660 at 39–43 °C using a 2–4 M NaOH solution for maintaining the pH. Due to an urgent need of material at an earlier stage of our work, a 153 kg batch experiment was run successfully under unoptimized conditions: 22% (w/w) initial substrate concentration, 7.3 kg Optimase® M 440, pH 7.5, 2 M NaOH, 47 h. The product isolation was based simply on repetitive extraction steps at neutral and acidic pH. On this large scale obstinate emulsion problems complicated work-up and suggested the use of centrifugation as an efficient means for fast and complete phase separation. Thus, a disk separator was chosen for a subsequent 2-kg experiment as described in the Experimental. Since such a separator can be operated continuously, it is suitable also for large-scale operations.

The racemic resolution of ester **3** was different from that of **1**: unlike **1** ester **3** did not melt upon warming of the reaction mixture (mp 75–76°C) and remained suspended in the aqueous buffer. Therefore, a two-phase-liquid system with **3** dissolved in a water-immiscible organic cosolvent was investigated.

Again, the different commercially available preparations of subtilisin Carlsberg were tested employing the enzymes as purchased in 10% amount (w/w or v/w) with respect to the starting ester. The time course for the reaction with the various enzyme preparations in aqueous suspension or aqueous/non-aqueous two-liquid-phase media, respectively, is shown in Figure 4. Following at first a procedure analogous to that for **1**, hydrolysis of **3** with Optimase® in aqueous sodium phosphate buffer (pH 7.5, 40 °C) afforded **4** in 46 % yield and >99 % *e.e.* within 46 h, even though **3** was suspended in the aqueous medium. Exchange of solid Optimase® M 440 with liquid Protease® L 660 or Alcalase®L 2.5 similarly provided **4** nearly quantitatively and with >99 % *e.e.* However, in comparison to Optimase®, the reaction with Protease® L 660 ran at a slower rate, and with Alcalase®L 2.5 it could only be driven to completion by multiple addition of the enzyme.

Work-up of the aqueous reaction mixtures was carried out by removal of unhydrolyzed (*R*)-**3** by filtration and extraction, acidification of the aqueous filtrate and product extraction (preferably with ethyl acetate), but was accompanied in the second extractive step by persistent emulsions caused by a film of denatured enzyme after acidification. In addition to this, work-up of the Optimase® M 440 reaction mixture was further complicated by very fine insoluble enzyme additives. These problems seriously limited the utility of this procedure for large scale-preparation with conventional batch extraction but, again, they could be overcome by using a disk separator (Experimental Section).

On the 200 g-scale an alternative procedure was worked out with the liquid Protease® L 660, promising facilitated work-up as compared to Optimase®, not needing a separator. The rate of hydrolysis was clearly improved by running the Protease® L-catalyzed reaction in an aqueous/organic two-phase-liquid system with a *tert*-butyl methyl ether (TBME) solution of **3**. Complete conversion was reached in about the same time as for the Optimase® reaction (Figure 4) without loss of enantioselectivity (>99 % *e.e.* before crystallization; the same result was obtained with Alcalase®2.5 L). Unreacted (*R*)-**3** was easily removed by separating the TBME layer. Denatured enzyme preventing phase separation in the batch-extraction of **4** with ethyl acetate was efficiently removed by filtration with the filter aid Dicalite Speedex®. Final purification was achieved by crystallization from ethyl acetate/ *n*-hexane and afforded **4** in 46 % yield and >99 % *e.e.* (see Experimental). To test the ruggedness of the two-liquid phase procedure we determined the effect of pH on the enantiomeric excess of crude **4** after a fixed reaction time of 65 h. Analogous to previous results, enzymatic hydrolysis at pH 7.5 and 8.5 provided **4** in excellent enantiomeric purity (>99 % ee). At pH 9.5 enantiomeric purity of **4** (98.5 % ee) was slightly impaired due to the expected chemical hydrolysis of racemic **3**.

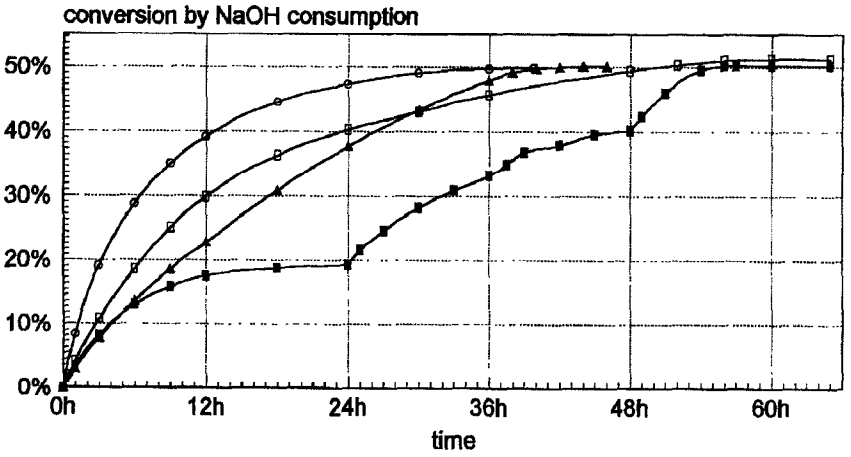

Figure 4. Time course for hydrolysis of racemic **3** at 40 °C with different industrial subtilisin preparations: (a) in aqueous sodium phosphate buffer, pH 7.5, with Optimase® M 440 (▲); with Protease® L 660 (□); with Alcalase® 2.5 L (three portions, ■). (b) in *tert*-butyl methyl ether/aqueous sodium phosphate buffer, pH 7.5, with Protease® L 660 (○).

Several experiments with 17 kg of **3** were successfully carried out, still using the earlier procedure with Optimase®. For the biphasic approach no explosion-proof facilities were available.

Determination of the enantiomeric excess

Several chiral GC columns were unsuccessfully examined for direct separation of the methylated enantiomers. Therefore, the product acids had to be separated as diastereomers using (*S*)-1-phenylethylamine as derivatization agent. The derivatization reaction described in the Experimental had proven to proceed quantitatively without discrimination of one diastereoisomer (Figure 5).

Discussion

An efficient enzymatic process was developed for the preparation of the (*S*)-configurated acids **2** and **4** which is also suitable for application on a large scale. The obstacle of the poor substrate solubility could be overcome by conducting the reaction above the melting temperature of the substrate (**1**) or by dissolving the substrate (**3**) in a water-immiscible cosolvent. These conditions could be applied due to the extraordinary properties of the employed enzyme: subtilisin Carlsberg turned out to be extremely tolerant to high concentrations of substrate, salt and organic solvent, *c.f.*[14,18,21,30] to elevated temperature or to a combination of all, respectively. It displayed its excellent enantioselectivity at an acceptable reaction rate even under such drastic and unphysiological conditions as an initial substrate concentration of 50 % (w/w) or 33 % using 4 M NaOH as titrating agent at pH 9. Because of the very low price of this detergent enzyme, its immobilization[*c.f.* 17,30] was not considered, the more as potential advantages like reduced emulsion problems during work-up are expected to be exchanged for disadvantages such as mass transfer limitations, and since the emulsion problems could be overcome by suitable measures (see below). Vigorous stirring was crucial for high hydrolysis rates, however, the formation of foam inactivated the enzyme in a reversible manner. In one case, upon formation of a foam cap the activity was lost almost completely but was fully restored as the foam was driven back by overpressure and gentler stirring. Therefore, on a larger scale the reactions were conducted under slight nitrogen overpressure (0.1–0.3 bar) helping to suppress foam formation. The simple separation of the reaction products also contributes to the attractiveness of this enzymatic process. Once the emulsions could be handled using a separator or the described biphasic system, the work-up became routine. A major disadvantage of the present process is the unsatisfactory recycling of the unwanted isomer.

A very straightforward alternative route towards remikiren would have been the resolution of **1** by direct aminolysis with histidine ethylester (*c.f.* Figure 2) and not by hydrolysis. Thus, we tested aminolysis of ester **1** using Alcalase® in EtOH or *t*BuOH according to a procedure by Chen *et al.*,[31] however, without success.

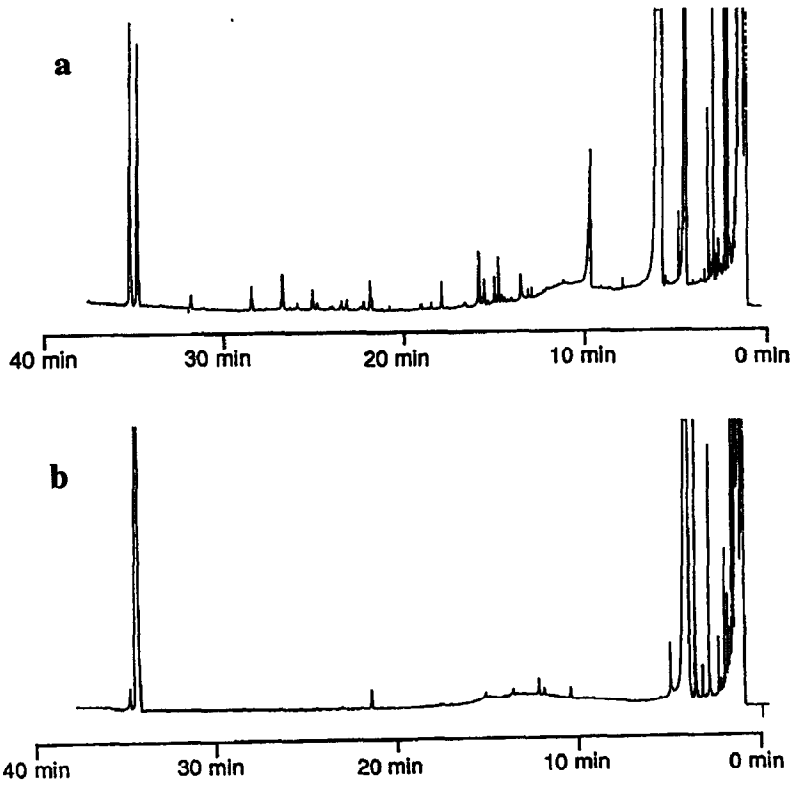

Figure 5. Determination of the enantiomeric excess of racemic 2-benzyl-3-(*tert*-butylsulfonyl)propionic acid by separation of its diastereoisomeric (*S*)-1-phenylethylamine derivatives on GC: (a) racemate, (b) (*S*)-acid **2**.

Experimental

General

The conversion degree was determined according to the consumption of NaOH solution of defined concentration (back titration).[32] NMR spectra were recorded on a Bruker AC (250 MHz) or AM (400 MHz), IR spectra on a Nicolet-FT IR 20 SXB. EI-MS were measured on an MS 9 AEI updated with VG console. Optical rotations were determined on a Perkin Elmer Polarimeter 241, and melting points (uncorrected) on a Büchi 530 or Mettler FP5 apparatus. GC determinations were carried out on a HP-5890-II.

Materials

Sulfopropionylesters **1** and **3** were prepared as described by Bühlmayer *et al.*[7] or Branca *et al.*,[3] respectively, and were of ≥99 % purity; bulk quantities were purchased from Robinson Brothers Ltd, West Bromwich, UK. Optimase® M 440 (440,000 DU/g·min), Protease® L 660 (660,000 DU/mL/min) and HT Proteolytic® 200 (200 NU/g) were purchased from Miles Kali-Chemie (now renamed as Solvay Enzymes, Hannover, Germany). Alcalase®2.0 T (2.0 AU/g) and 2.5 L (2.5 AU/g) as well as Savinase® 6.0 T (6.0 KNPU/g) were obtained from Novo Nordisk (Bagsvaerd, DK) and Prozyme® 6 (60000 U/g) from Amano (Nagoya, Jpn). α-Chymotrypsin and (*S*)-1-phenylethylamine were purchased from Fluka. Solvents were of technical grade.

Enantioselective hydrolysis of 1 using a disk separator for work-up

Ester **1** (2.00 kg, 6.40 mol) was emulsified in 4 L of 13 mM borax–HCl buffer pH 9 of 40 °C by melting and vigorous stirring. After addition of 200 mL Solvay Protease® L 660 the pH-drop was immediately readjusted to 8.5 and pH then kept constant (pH-stat) by the addition of 2.0 M NaOH under vigorous stirring at 39–40 °C. After completion of the reaction (1586 mL, 49.5% conversion, 42.3 h) the reaction mixture (8 L) was cooled down to room temperature. (*R*)-**1** was removed by extraction three times with ethyl acetate (8 L, 4 L, 4 L). Phase separation occurred spontaneously within 10 min. The aqueous solution was then adjusted to pH 2 by addition of 98 % H_2SO_4 (105 mL). After extraction with ethyl acetate (8 L) the emulsion formed was separated on a disk separator (Westfalia SA-1) operated at 7900 rpm (4900 *g*). Complete phase separation was attained at a flux of 22–28 L/h. After the second extraction with ethyl acetate (5 L) the emulsion separated spontaneously. The combined organic phases were washed with deionized water (5 L), concentrated to 2.5 L, and residual water was removed (< 1 %) by azeotropic distillation with ethyl acetate (5 x 0.5 L). Crystallization of **2** was achieved by adding *n*-hexane (4 L) to the 2.5 L of solution with vigorous stirring. After crystallization had started further *n*-hexane (2 L) was added and the suspension stirred at 4 °C for 1.5 h. The crystals were filtered off, washed with 1 L of *n*-hexane and dried *in vacuo* at 40 °C to provide 760 g (42 %) of **2** as colourless crystals:[33] mp 99.5–100.5 °C; $[\alpha]_D$ = +12.9 ° (*c* = 1 %, $CHCl_3$); $[\alpha]_{365}$ = +60.6 ° (*c* = 1 %, $CHCl_3$); GC ≥ 99 % *e.e.*; ^{1}H NMR 400 MHz, ($CDCl_3$): δ 1.36 (s, 9H, CH_3), 2.94–3.08 (m, 2H, CH_2Ph, CH_2SO_2), 3.20–3.25 (m, 1H, CH_2), 3.40–3.49 (m, 2H, CH_2CHCH_2, CH_2), 6.9 (bs, COOH), 7.19–7.39 (m, 5H, Ph-H); MS (EI): m/z (%) 284 (0.2, M^+), 57 (100); IR (KBr): 1708 cm^{-1} (COOH); Anal. calcd for $C_{14}H_{20}O_4S$ (284.37): C, 59.13; H, 7.09; S, 11.27; found: C, 59.20; H, 6.93; S, 11.20.

Enantioselective hydrolyses of 3 in a TBME/aqueous buffer two-phase-liquid system

Compound **3** (200.0 g, 481 mmol, 99.0 % purity) was dissolved in a mixture of 600 mL TBME and 1400 mL of 1.9 mM sodium dihydrogenphosphate at 40 °C. The biphasic solution was adjusted to pH 7.5 with 1 M NaOH. The reaction was started by adding 20 mL Protease® L 660 and the pH maintained at 7.5 by addition of 1.0 M NaOH (pH-stat device) with stirring at 40 °C. After consumption of 243–247 mL of 1.0 M NaOH (50.5–51.4 % conversion,[32] after 65 h) the mixture was cooled to room temperature and the phases were separated. The organic layer was extracted with water (200 mL) and the combined aqueous phases were washed with TBME (300 mL). After addition of 16.0 g Dicalite Speedex® the aqueous solution was acidified to pH 1.5 with 3 M HCl (100 mL), ethyl acetate (800 mL) was added and after stirring for 5 min the resulting mixture was filtered. After phase separation the aqueous layer was extracted twice with ethyl acetate (400 mL, 200 mL). The combined organic phases were washed with water (200 mL), heated to reflux (400 mbar, 70 °C bath temperature, 4 h) for azeotropic removal of water and concentrated to about 300 mL. Crystallization was initiated under stirring at 46 °C by seeding and *n*-hexane (230 mL) was added in 30 min. The suspension was cooled to -10 °C and stirred for 2 h. The precipitated product was filtered off, washed with *n*-hexane (80 mL) and dried *in vacuo* to yield 85.1–85.4 g (46 %) **4** as yellowish white crystals: mp 121–122 °C; $[\alpha]_D$ = - 2.2 ° (c = 1 %, $CHCl_3$); $[\alpha]_{365}$ = +7.5 ° (*c* = 1 %, $CHCl_3$); GC ≥ 99 % *e.e.*; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.69 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 2.90–3.02 (m, 1H, CH_2Ph), 3.12–3.26 (m, 2H, CH_2Ph, CH_2SO_2), 3.32–3.47 (m, 1H, CH_2CHCH_2), 3.58–3.77 (m, 9H, morpholinyl-H, CH_2SO_2), 7.19–7.39 (m, 5H, Ph-H), 9.8 (bs, COOH); MS (EI): m/z (%) 383 (7, M^+), 157 (100); IR (KBr): 1751 cm^{-1} (COOH); Anal. calcd for $C_{18}H_{25}NO_6S$ (383.47): C, 56.38; H, 6.57; N, 3.65; S, 8.36; found: C, 56.25; H, 6.66; N, 3.53; S, 8.39.

Enantioselective hydrolysis of 3 suspended in aqueous buffer using a disk separator for work-up

Compound **3** (17.01 kg, 41.35 mol) was suspended in a solution of 54 g sodium dihydrogenphosphate dihydrate in 180 L of deionized water at 40 °C under vigorous stirring. The pH was adjusted to 7.5 by adding 2 M NaOH. The reaction was started by the addition of 765 g Optimase® M 440 (additional 340 g of enzyme after 30 h) and the pH maintained at 7.5 under vigorous stirring at 40 °C by the controlled addition of NaOH solution (1.96 mol/kg). After

consumption of 10.653 kg titrating solution (50.5% conversion)[32] the reaction mixture was adjusted to pH 8.0 with 28% NaOH (50 mL). Ethyl acetate (240 L) was added and the resulting emulsion stirred for 10 min. The mixture was then pumped through a disk separator (Westfalia SA-20) operated at 6500 rpm (9000 *g*). A flux of 300 L/h was maintained to obtain complete phase separation. The aqueous phase was extracted a second time as described with ethyl acetate (120 L) in order to remove (*R*)-**3** completely. The aqueous phase was set to pH 2 with 98 % H_2SO_4 (850 mL) and then stirred with ethyl acetate (110 L) for 10 min. The mixture was separated using the separator as described above. The aqueous phase was extracted a second time with ethyl acetate (145 L). Washing the combined ethyl acetate extracts three times with 20 % (w/v) NaCl solution (30 L each), followed by drying over anhydrous sodium sulfate (10 kg) and evaporation afforded 6.84 kg of **4** (43 %; >99 % *e.e.*) .

Determination of the enantiomeric excess

Derivatization. Compound **2** or **4** (0.1–1 mg) was dissolved in 1 mL CH_2Cl_2. Catalyst 2-chloro-1-methylpyridinium iodide (1 mg), 10 µL of tributylamine and 20 mg (*S*)-1-phenylethylamine were added and the mixture warmed for 30 min at 60 °C.[34] The reaction mixture was washed with 3 x 1 mL 2 N HCl, once with 1 mL distilled water and once with saturated NaCl solution. The organic phase was dried over Na_2SO_4 to obtain a solution ready for GC-injection.

GC-conditions. Column: fused silica, 25 m, i.d. 150 µm; stationary phase: UCW-98; carrier gas: H_2 at 50 $cm \cdot s^{-1}$; temperature: 180–280°C at 2 $°C \cdot min^{-1}$ for **2** and 200–290 °C at 1 $°C \cdot min^{-1}$ for **4**; injector: 280 °C; detector: 300 °C.

Acknowledgement

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