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Lecture Transcripts

Process Development on the Enantioselective Enzymatic Hydrolysis of *S*-Ethyl 2-Ethoxy-3-(4-hydroxyphenyl)propanoate

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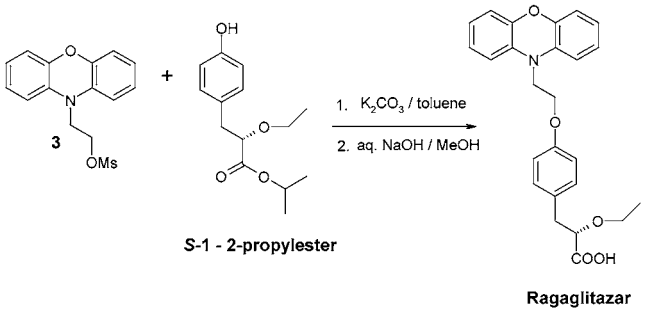
Abstract:

A novel biocatalytic approach for the large-scale production of *S*-2-ethoxy-3-(4-hydroxyphenyl)propanoic acid *S*-1 from its racemic ethylester *rac*-2 by enantioselective hydrolysis has been developed. *S*-1 is an important building block in the synthesis of PPARα and -γ agonists such as Ragaglitazar [NNC 61-0029 ((−)DRF2725)]. The development history comprises enzyme screening, biocatalyst and process optimization, and scale-up to pilot plant. The project was thereby highly interdisciplinary by combining biotechnology and chemistry technologies. The final process was successfully run on a 44-kg pilot scale in 43−48% yields and with high enantiomeric purities (98.4−99.6% ee).

Introduction

S-2-Ethoxy-3-(4-hydroxyphenyl)propanoic acid ***S*-1** is a key intermediate in the synthesis of the new antidiabetic drug Ragaglitazar [NNC 61-0029 ((−)DRF2725)].[1,2] Ragaglitazar [NNC 61-0029] belongs to a novel therapeutic class of compounds of dual acting PPARα and -γ agonists aimed for treatment of type-2 diabetes.[3] Ragaglitazar is designed to restore insulin sensitivity and to correct the dyslipidaemic disorders in people with type-2 diabetes.[4,5] The compound, in-licensed by Novo Nordisk from Dr. Reddy's Laboratories, is chemically and pharmacologically different from presently marketed PPAR agonists and is a member of a new class of insulin sensitisers. Ragaglitazar [NNC 61-0029] has currently entered phase III clinical trials for treatment of diabetes mellitus type 2.

Scheme 1. **Synthesis of Ragaglitazar [NNC 61-0029 ((−)DRF2725)]**



Ragaglitazar [NNC 61-0029] can be prepared by the condensation of 2-[phenoxazin-10-yl]ethylmethane sulphonate **3**[6] with the 2-propylester of ***S*-1** in the presence of potassium carbonate in refluxing toluene. The acid ragaglitazar [NNC 61-0029] can then be obtained by basic hydrolysis of the coupled 2-propylester[4,7] (Scheme 1). The development for the synthesis of the key intermediate, enantiomerically pure ***S*-1**,[8] will be described in this work.[9,10]

A reported scalable synthetic route for the synthesis of enantiomerically pure ***S*-1** or its esters uses diazotation of O-benzylated L-tyrosine to afford its α-hydroxy acid, followed by alkylation, with high retention of the chirality.[1] However, this process had in the earlier preparations certain drawbacks such as moderate yields, the formation of about 20% bi-product, and up to 4% racemisation.[11]

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(1) Siripragada, M. R.; Chebiyyam, P.; Potlapally, R. K.; Batchu, C. S.; Mamillapally, R. S. PCT Appl. WO 00/26200.

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(5) Wulff, E. M.; Jeppesen, L.; Bury, P. S.; Mogensen, J. P.; Fleckner, J.; Andersen, A.-S. T.; Wassermann, K.; Sauerberg, P. *Diabetes* **2001**, *50* (Suppl. 2), A524.

(6) Batchu, C.; Mamillapalli, R. S.; Gaddam, O. R. PCT Appl. WO 02/34733.

(7) Despite the excellent results of the process described in this article, another synthetic pathway using different intermediates was chosen for further large-scale production.

(8) Other antidiabetic drugs in development use the same building block, stressing the importance of a straightforward synthesis of the title compound: Thalen, P.; Jacinto, S.; Hultstrand, T.; Amrot-Fors, L.; Camejo, G.; Wallin, B.; Svensson, L.; Brockenhuus, H.; Ljung, B.; Oakes, N. *Diabetes* **2001**, *50* (Suppl. 2), A122; Murphy, G. J.; Holder, J. C. *Trends Pharmacol. Sci.* **2000**, *21*, 469−474.

(9) Ebdrup, S.; Deussen, H.-J.; Zundel, M.; Bury, P. S. PCT-Appl. WO 01/11073.

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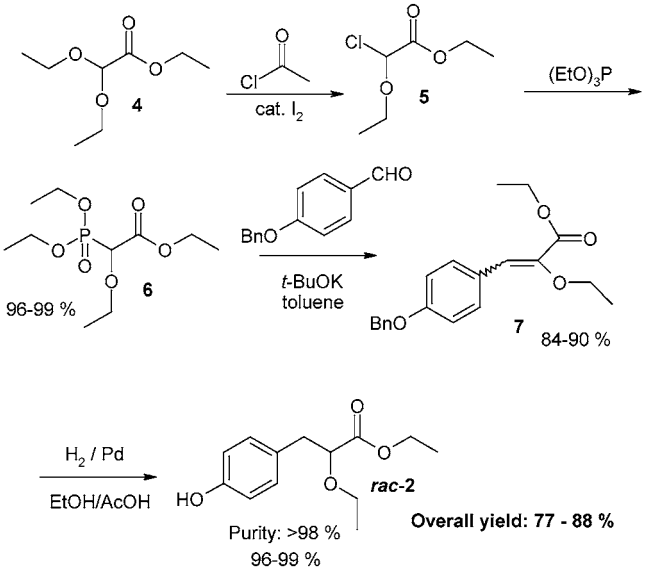
Scheme 2. **Pilot-plant synthesis of *rac*-2**

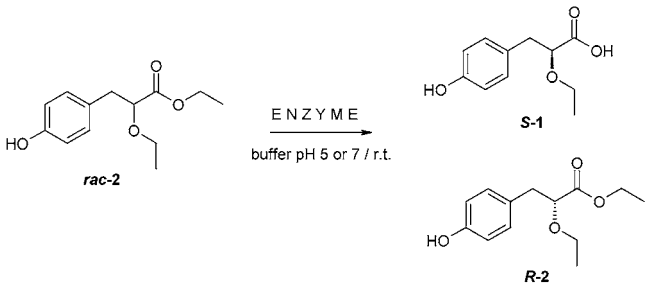


Biocatalysis has become a widely used tool in the pharmaceutical industry to the synthesis of optically active intermediates.[12] Enantioselective hydrolysis of esters and enantioselective esterification of carboxylic acids using hydrolases are among the most commonly used methods for the preparation of optically pure alcohols and esters. This is due to the fact that hydrolases do not require cofactors, are simple to use, and are now widely commercially available.[13] It is known from the literature that substituted α-benzyloxypropanoates[14] and α-methoxypropanoates[15] can be enantioselectively hydrolyzed to their corresponding carboxylic acids by biocatalysis. Athough the first method uses growing cells and the second method only discloses moderate enantioselectivities, these results encouraged us to search for an enzymatic kinetic resolution for the large-scale preparation of enantiomerically pure ***S*-1** or an ester of ***S*-1** with a target enantiomeric excess of >98% ee. This work includes screening for enantioselective enzymatic activity, process and biocatalyst optimization, as well as scale-up to pilot plant, which all will be described herein.

Results and Discussion

Synthesis of *rac*-Ethyl 2-Ethoxy-3-(4-hydroxyphenyl)-propanoate (*rac*-2). The synthesis of racemic ethyl 2-ethoxy-3-(4-hydroxyphenyl)propanoate ***rac*-2** is depicted in Scheme 2. Commercially available 2,2-diethoxyacetate **4** was chlorinated with 1.2 equiv of acetylchlorid using a catalytical amount of iodine to give 2-chloro-2-ethoxyacetate **5**.[16] Toluene was added to the reaction mixture, and low-boiling components were distilled off together with the toluene.

Scheme 3. **Enantioselective enzymatic hydrolysis of *rac*-2**



The reaction mixture was then heated with triethyl phosphite to 150 °C[17] to give triethyl 2-ethoxyphosphonoacetate **6** in 96−99% yield (over both steps). **6** was reacted with 4-benzyloxybenzaldehyde in a Horner−Emmons−Wadsworth reaction[18] using *t*-BuOK as a base. 3-(4-Benzyloxyphenyl)-2-ethoxyacrylic acid ethyl ester **7** could be obtained by crystallization from the reaction mixture as an *E*/*Z*-mixture. Hydrogenation of **7** at 2−6 bar yielded ***rac*-2** in 77−88% overall yield in >98% purity.[4,19] All steps were successfully scaled-up to pilot plant.

Enzyme Screening. More than 80 hydrolases (lipases, esterases, cutinases, proteases) were screened for enantioselectivity on **2**[20] as a substrate in two screening rounds (Scheme 3). The initial screening revealed three enzyme samples to be highly enantioselective for the *S*-enantiomer[21] with an enantiomeric ratio[22] $E > 200$: protease 2 (aspergillopepsin I) from *Aspergillus aculeatus*, alp protease (Oryzin) from *Aspergillus oryzae*,[23] and proteinase 2A from *Aspergillus oryzae* (Fluka).

However, when the hydrolysis was repeated using highly purified samples of protease 2, no activity at all was found. The active enzyme preparation used in the first screening was shown to contain traces of other secreted proteins from the production organism, suggesting that one of these proteins was the enzyme responsible for the observed catalytic activity. Therefore, a number of additional enzymes, with a focus on commercially available industrial enzyme preparations produced by the two *Aspergillus* strains including *Aspergillus niger* were tested in a second screening round. The results (selected are listed in Table 1) revealed the desired enzymatic activity to be present in all *Aspergillus* preparations tested.[24] Although the enantioselectivities of many of the enzyme preparations were very high, the specific activities (hydrolytic activity towards ***S*-2**/g of enzyme

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(17) Grell, W.; Machleidt, H. *Justus Liebigs Ann. Chem.* **1966**, 53−67.
(18) Wadsworth, Jr. *Org. React.* **1977**, *25*, 73−253.
(19) Enantioselective hydrogenation of a 36/64 *E*/*Z* mixture resulted in only 45% ee (100% *Z*: 85% ee) for the best catalyst tested.
(20) Other esters of ***S*-1** (2-propyl, hexyl, 2-ethoxyethyl) were included in the initial screening to elucidate a potential influence of the chain length on the enantioselectivity. Differences in enantioselectivity found were only within experimental and analytical error.
(21) An enzyme which is selective towards the *R*-enantiomer would be preferable, but only two enzymes tested were *R*-selective with an enantiomeric ratio of $E < 6$ (α-chymotrypsins from bovine pancreas).
(22) Chen, C.-S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294−7299.
(23) Experimental samples from Novozymes A/S, Denmark.
(24) The low enantiomeric ratio *E* found for Flavourzyme can be explained by its high content of substrate unspecific proteases/peptidases.

Table 1. **Screening results: enantiomeric ratios (*E*) for selected enzymes**

enzyme	enantiomeric ratio (*E*)
protease 1 (aspergillopepsin II from *A. aculeatus*)[a,c,d,f]	18
protease 2 (aspergillopepsin I from *A. aculeatus*)[a,c,d,f]	>200
NpI protease (neutral proteinase/fungalysin from *A. oryzae*)[a,c,f]	71
protease 2A[b,c,f]	>200
alp. protease (oryzin from *A. oryzae*)[a,c,f]	>200
Flavourzyme[a,c]	4
Pectinex BE 3L[a,e]	104
Pectinex ultra SP-L[a,c,d]	>200
Kojizyme 500 mg[a,c]	>200
Shearzyme 500 L[a,c]	>200
ferulic acid esterase[a,c]	>200
Pectinex SP-L[a,d]	>200
Rheozyme[a] (pectin methyl esterase)[a,d,c]	>200
Pectinex AFP L-2[a,d,e]	>200
Novozyme 188[a,e]	>200
acetyl xylan esterase[a,,c,d]	>200

[a] Novozymes. [b] Fluka. [c] Expressed in *Aspergillus oryzae*. [d] Expressed in *Aspergillus aculeatus*. [e] Expressed in *Aspergillus niger*. [f] Screened in the first round (all other enzymes were screened in the second round).

preparation) were rather low, strongly suggesting that the observed activity originated from a secreted enzyme present in these crude biocatalyst preparations only in small concentrations.

First Laboratory Scale-Up. The first scale-up of the enzymatic hydrolysis was performed using Protease 2A from Fluka. This enzyme showed the best compromise between activity and enantioselectivity among the first enzymes screened, which were available on a larger scale.[25] A number of small-scale experiments aimed at optimizing the enzymatic hydrolysis on a milligram−gram-scale of the enzymatic hydrolysis were performed.[26] These experiments revealed large batch-to-batch variations in activity and enantioselectivity of the Fluka enzyme. The best procedure was then run on a 70-g scale in an 18-L separatory funnel with mechanical stirring; Table 2 lists the characteristics of the reactions.

In both trials, the substrate was dissolved in buffer and stirred at room temperature with the enzyme. The pH was kept around 7 by addition of 30% aqueous NaOH, where the consumed base correlates with the degree of conversion. The unreacted ester was then removed by extraction after 45% conversion. The aqueous reaction mixture left was then acidified to pH 2−3 by addition of hydrochloric acid, followed by extraction of ***S*-1**. During those extractions emulsions formed (due to the high content of biocatalyst), which resulted in incomplete phase separations (even after allowing to settle for days). Attempts to break the emulsions by filtration through a variety of filter aids or by heating remained unsuccessful. Nevertheless, filtering of the reaction mixtures through sand partially broke the emulsions, which then were further allowed to settle for days. The organic phases were finally separated and collected. This procedure was repeated three to four times. The combined organic phases were dried over Na_2SO_4, and ***S*-1** could be isolated in good yields as an oil after evaporation of the solvent. The experiences from this first scale-up (enzyme batch-to-batch variations, emulsions during workup caused by the biocatalyst preparation, high substrate dilution) suggested that the biocatalyst preparation should be improved and that development work was needed on the process.

Biocatalyst Development. To get a more selective and active biocatalyst preparation, the most straightforward and fastest approach was to optimize the fermentation of *Aspergillus oryzae* for the production of the enzyme of interest. The medium pH was found to be a critical process parameter and had to be kept at 4.5 throughout the fermentation. Using the improved fermentation process, three fermentations were run on a pilot scale (1300-L fermentor). The fermentation supernatant obtained in a filtration step was concentrated by removal of water in an ultrafiltration. One of the ultrafiltrates was furthermore freeze-dried followed by homogenization; 1.9 kg (lot A) of a freeze-dried biocatalyst powder and two liquid enzyme preparations (lot B, 24 kg; lot C, 20 kg) were produced, which were used for the further development described in this article.[27]

Process Development. To define the optimum process conditions, the influence of reaction parameters such as temperature, pH, co-solvent addition (type of solvent and phase ratio), substrate concentration, and biocatalyst loading on the reaction rate and enantioselectivity of the hydrolysis of ***S*-2** was investigated using the new biocatalyst batches A−C. It was found to be most advantageous to run the reaction at a temperature of 20−30 °C (preferably 25 °C), keeping the pH between 6.5 and 7.5 (automated titration with 30% NaOH).[28] Outside this range, both activity and enantioselectivity decreased. The addition of cosolvents (to increase the solubility of the substrate), such as acetone, THF, ethanol, or 2-propanol resulted in prolonged reaction time and in reduced enantioselectivity. Instead of cosolvent addition to increase the poor water solubility of the substrate (∼5 g/L), the reaction was run as a biphasic emulsion in aqueous buffer. It was found that reducing the amount of buffer negatively affected the reaction rate. The ratio between substrate and buffer was therefore investigated to optimize space−time yields and the ease of workup.

Even though the amount of biocatalyst could be significantly reduced compared to that used in the first laboratory scale-up reactions, due to higher enzymatic activities, emulsions still formed at acidification to pH 2−3 during workup. Again, all commonly employed methods, such as the usage of different filter aids to break the emulsions and to

(25) Only the results from the first screening round were known at this time. Due to the urgent demand of the project ***S*-1** was produced on the basis of the best knowledge available. The results obtained by the second screening round together with additional lab-scale experiments obtained later suggest Pectinex Ultra SP-L as the most favorable enzyme mixture.

(26) *Biocatalysis for Fine Chemical Synthesis*; Roberts, S. M., Ed.; John Wiley & Sons: Chichester, 1999.

(27) The relative activities/g of enzyme preparation were determined by activity assay as following: lot A/B/C: 8.4/1.3/1.0. The enzymatic activity present in each batch hydrolyzed ***rac*-2** with an enantiomeric ratio $E > 200$.

(28) Nonselective chemical hydrolysis was <0.1% after 4 days while stirring in buffer without enzyme applying these conditions.

Table 2. **Characteristics of the first laboratory scale-up using proteinase 2A from Fluka**

	first trial	second trial
scale (***rac*-2**)	70 g	70 g
proteinase 2A	52 g	10 g
phosphate buffer pH = 7/0.1 M	200 mL/g, ***rac*-2** (solution)	200 mL/g, ***rac*-2** (solution)
reaction time	8 h	48 h
conversion	**45%**	**45%**
total solvent used during	57 mL AcOEt/g ***rac*-2**	71 mL MTBE/g ***rac*-2**
pH 7 extractions	(3 extractions needed)	(4 extractions needed)
total solvent used	51 mL AcOEt/g ***rac*-2**	83 mL MTBE/g ***rac*-2**
during pH 3 extraction	(3 extractions needed)	(3 extractions needed)
comments	emulsion at pH 2−3	emulsion at pH 2−3
product isolation	drying of combined organic phases over Na_2SO_4 followed by filtration and evaporation of the solvent	drying of combined organic phases over Na_2SO_4 followed by filtration and evaporation of the solvent
isolated yield *S*-1	**43%**	**43%**
HPLC purity	**>96%**	**>96%**
% ee	**96.8**	**94.3**
calculated *E*	160	75

Table 3. **Characteristics of the improved laboratory procedure using the three hydrolytic enzyme mixtures (lots A−C) obtained by an optimized fermentation of *Aspergillus oryzae***

	lot A	lot B	lot C
scale (***rac*-2**)	50 g	50 g	50 g
Aspergillus oryzae hydrolytic enzyme mixture	0.8 g, lot A	8 g, lot B	15 g, lot C
phosphate buffer pH = 7/0.1 M	2 mL/g ***rac*-2** (biphasic mixture)	2 mL/g ***rac*-2** (biphasic mixture)	2 mL/g ***rac*-2** (biphasic mixture)
reaction time	30 h	23 h	25 h
conversion	**48%**	**45%**	**41%**
water removed by distillation	75%	75%	75%
methanol added	2.5 times the volume of the removed water	2.5 times the volume of the removed water	2.5 times the volume of the removed water
comments	precipitation and removal of the biocatalyst in aqueous methanol prior to extraction expeditious phase separation at pH 3.	precipitation and removal of the biocatalyst in aqueous methanol prior to extraction expeditious phase separation at pH 3.	precipitation and removal of the biocatalyst in aqueous methanol prior to extraction expeditious phase separation at pH 3.
total solvent used during pH 7 extractions	5 mL MTBE/g ***rac*-2** (3 extractions needed)	5 mL MTBE/g ***rac*-2** (3 extractions needed)	6 mL MTBE/g ***rac*-2** (3 extractions needed)
total solvent used during pH 3 extraction	4 mL MTBE/g ***rac*-2** (3 extractions needed)	4 mL MTBE/g ***rac*-2** (2 extractions needed)	4 mL MTBE/g ***rac*-2** (2 extractions needed)
product isolation	drying of the combined organic phases over Na_2SO_4 followed by filtration and evaporation of the solvent	drying of the combined organic phases over Na_2SO_4 followed by filtration and evaporation of the solvent	drying of the combined organic phases over Na_2SO_4 followed by filtration and evaporation of the solvent
isolated yield *S*-1	**48%**	**40%**	**41%**
HPLC purity	**>99%**	**>98%**	**>98%**
% ee	**99.1**	**99.3**	**99.6**
calculated *E*	>200	>200	>200

improve phase separations, were unsuccessful. We then discovered that both the acid **1** and the ester **2** were soluble in aqueous methanol[29] (pH 7), while the biocatalyst formed a filterable precipitate under the same conditions. In a typical process, 75% of the water from the reaction mixture was evaporated, and methanol was then added (volume of methanol added: 2.5−4.0 times the volume of the water previously removed[30]) to solubilize both substrate and product[31] while precipitating the biocatalyst. Cellulose powder from spruce proved to be a very efficient filter aid to accelerate the following filtration. After the biocatalyst precipitate had been removed, the methanol was distilled off, and the original amount of water was added again. The following extractions with MTBE, at neutral pH and after acidification to pH 2−3, did not give emulsions and resulted in expeditious phase separations. A series of experiments were run on a 50−100-g scale to adjust the reaction parameters and the workup procedure. Table 3 lists typical characteristics of the process using the three different enzyme lots A−C.

In comparison to the first scale-up, the new laboratory procedures were now significantly improved: The reagent concentration was significantly higher, less solvent was used

(29) Ethanol or 2-propanol can be used as well.

(30) The ease of filtration of the precipitate improved, when the amount of methanol was increased. Usually the phase separations in the following steps were also faster with increasing amounts of methanol.

(31) The conversion was most precisely determined by HPLC analysis of this solution.

Table 4. **Characteristics of the semi-pilot batches**

	first semi-pilot batch	second semi-pilot batch
scale (***rac*-2**)	15.1 kg	13.8 kg
Aspergillus oryzae	0.27 kg, lot A	0.22 kg, lot A
hydrolytic enzyme mixture	(additional 84 g enzyme added after 22 h)	
phosphate buffer	2.5 mL/g ***rac*-2**	2.9 mL/g ***rac*-2**
pH = 7/0.1 M	(biphasic mixture)	(biphasic mixture)
reaction time	27 h	30 h
conversion	**42%**	**44%**
water removed by distillation	90%	85%
methanol added	2.0 times the volume of the removed water	2.3 times the volume of the removed water
removal of biocatalyst	filter (4.5 h)	(centrifuge 40 min)
comments	expeditious phase separation at pH 2−3.	expeditious phase separation at pH 2−3.
total solvent used	6 mL MTBE/g ***rac*-2**	4 mL MTBE/g ***rac*-2**
during pH 7 extractions	(4 extractions needed)	(3 extractions needed)
total solvent used	3 mL MTBE/g ***rac*-2**	3 mL MTBE/g ***rac*-2**
during pH 3 extraction	(2 extractions needed)	(2 extractions needed)
product isolation	concentrated under vacuum, 2-propanol added and distilled off until <0.2% water	concentrated under vacuum, 2-propanol added and distilled off until <0.2% water
isolated yield *S*-1	**41%**	**42%**
HPLC purity	**97.8%**	**98.6**
	1.3% methyl ester	**0.6% methyl ester**
	0.7% 2-propyl ester	**0.8% 2-propyl ester**
% ee	**98.7**	**98.6**
calculated *E*	>200	>200

for the extraction procedures, the workup was now scalable to pilot-plant scale (batch reactor), and chemical and enantiomeric purities were improved. The results also showed that enantiomeric purities >99.0% ee should be obtainable at 45% conversion with these enzyme batches. Two kilo-lab batches were produced using enzyme lot A, on a 500- and 1000-g scale, showing the robustness of the process.[32]

Scale-Up to Pilot. Some changes were introduced during the technology transfer to semi-pilot to adapt the process to plant requirements: The reaction mixture was heated to 80−90 °C after the desired conversion of 45%. At that temperature the biocatalyst irreversibly deactivated. This excluded the risk of exceeding hydrolysis above the desired conversion in case of prolonged workup and resulted in a more robust process.[33] It was technically possible to distill off more water from the reaction mixture than as initially judged during the laboratory optimization. Consequently, lower amounts of methanol were necessary to solubilize the product and the substrate, without influence on the filtration step using semi-pilot equipment. To accelerate the filtration, a centrifuge was used instead of a filter. The solvent volumes (MTBE) used for the extractions could be reduced without a negative impact on the phase separations. Finally, the combined MTBE solutions containing ***S*-1** were not treated with Na_2SO_4 followed by filtration as done in the laboratory procedures. Instead, the water was removed by azeotropic distillation with most of the MTBE. 2-Propanol was added to the reaction mixture, and the remaining MTBE/water was distilled off with some of the 2-propanol to give ***S*-1** as a 50−70% (weight) solution in 2-propanol. In addition to the advantage of shortening the process (omitting a filtration step), storing of ***S*-1** in solution (2-propanol is the solvent to be used in the next reaction step) avoided the risk of undesired crystallization.[34] Two semi-pilot batches were produced by applying these modifications, and the results are listed in Table 4. Recycled solvents were used for the second batch.

The product quality was not significantly changed in comparison to that in the laboratory trials. Some ***S*-1** methyl and 2-propyl ester were formed during the removal/distillation of the alcohols. As ***S*-1** will be transformed into the 2-propyl ester in the following reaction anyway, the presence of ester impurities was not an issue. The enantiomeric purities were somehow lower in comparison to the values usually observed during the laboratory trials but still above our target range (>98.0% ee).

The process was finally run in pilot-plant using all three enzyme lots. Table 5 lists the results of the pilot campaign.

Except that slightly more water was removed prior to the biocatalyst precipitation, the pilot-process conditions remained basically unchanged in comparison to those of the semi-pilot process. The reaction times were shorter in comparison to those in the laboratory and semi-pilot trials, probably due to a better mixing in the pilot reactor. While the phase separations during the MTBE extractions normally took between 30 and 60 min in all the earlier trials, unsatisfactory phase separations were observed in batches 1, 2, and 4 during the first pH-7 extractions (see Table 5). In semi-pilot the same enzyme was used as in the pilot first batch without any phase-separation problems. We do not see a reason for this behaviour in the pilot trial. More experience would be needed to identify critical parameters if the process were to be continued with similar enzyme batches. Chemical

(32) kg batch: 41% hydrolysis, 39% isolated yield, >99% HPLC purity, 98.9% ee; 500 g batch: 42% hydrolysis, 41% isolated yield, >99% HPLC purity, 98.8% ee.

(33) Tested on 50-g scale first.

(34) Pure ***S*-1** is a crystalline solid (mp = 105 °C). Usually the ***S*-1** obtained after the processes was obtained as an oil, which then slowly crystallized on standing.

Table 5. **Characteristics of the pilot batches**

	first pilot batch	second pilot batch	third pilot batch	fourth pilot batch
scale (***rac*-2**)	43.9 kg	43.9 kg	43.9 kg	43.9 kg
Aspergillus oryzae hydrolytic enzyme mixture	0.74 kg, lot A	7.5 kg, lot B	7.5 kg, lot B	7.5 kg, lot C
phosphate buffer pH = 7/0.1 M	2.5 mL/g ***rac*-2** (biphasic mixture)	2.5 mL/g ***rac*-2** (biphasic mixture)	2.5 mL/g ***rac*-2** (biphasic mixture)	2.5 mL/g ***rac*-2** (biphasic mixture)
reaction time	21 h	20 h	16 h	16 h
conversion	**43%**	**47%**	**44%**	**48%**
water removed by distillation	93%	93%	95%	94%
methanol added	2.3 times the volume of the removed water	2.3 times the volume of the removed water	2.3 times the volume of the removed water	2.3 times the volume of the removed water
comments	extraction at pH7: first layer separated overnight. second layer separated overnight only with additional MTBE. extraction at pH3: 30−60 min for all phase separations	extraction at pH7: first layer separated overnight. second and third layer separated within 30−60 min extraction at pH3: 30−60 min for all phase separations	extraction at pH7: first layer separated within 60 min second layer separated within 30 min extraction at pH3: 30−60 min for all phase separations	extraction at pH7: first layer separated over 2 days. second layer separated within 30−60 min extraction at pH3: 30−60 min for all phase separations
total solvent used during pH 7 extractions	5 mL MTBE/g ***rac*-2** (2 extractions needed)	5 mL MTBE/g ***rac*-2** (3 extractions needed)	3 mL MTBE/g ***rac*-2** (2 extractions needed)	3 mL MTBE/g ***rac*-2** (2 extractions needed)
total solvent used during pH 3 extraction	3 mL MTBE/g ***rac*-2** (2 extractions needed)	3 mL MTBE/g ***rac*-2** (2 extractions needed)	3 mL MTBE/g ***rac*-2** (2 extractions needed)	3 mL MTBE/g ***rac*-2** (2 extractions needed)
product isolation	as in semi-pilot	as in semi-pilot	as in semi-pilot	as in semi-pilot
isolated yield *S*-1	**43%**	**44%**	**44%**	**48%**
HPLC purity	**97.7%** **1.7% 2-propyl ester**	**98.4%** **0.7% 2-propyl ester**	**98.8%** **0.3% 2-propyl ester**	**99.3%** **0.1% 2-propyl ester**
% ee	**99.4**	**98.4**	**99.0**	**99.6**
calculated *E*	>200	>200	>200	>200

Table 6. **Comparison of the initial laboratory procedure, the pilot plant process described in this article, and a potential future process using the cloned enzyme**

	first laboratory scale-up	pilot-plant process	possible process using the cloned enzyme
enzyme/kg ***S*-1**	143−743 g (freeze-dried)	17 g (freeze-dried) or 170−340 g (ultra filtrate)	2 g (ultra filtrate)
MTBE/kg ***S*-1**	154 l	6−8 l	6−8 l
MeOH/kg ***S*-1**	none	5.8 l	none
water/kg ***S*-1**	200 l	5.0 l	2.5 l
Na_2SO_4/kg ***S*-1**	ca. 1 kg	none	none
filter aid (cellulose)	none	130 g	none
distillation steps	2	5	3
filtration steps	2	1	0

purities and isolated yields of the product ***S*-2** were again excellent. The enantiomeric purities were higher than in the semi-pilot campaign despite the fact that the hydrolyses were run to higher conversions.

Outlook

The combination of biocatalyst and process development described in this article allowed quick progress from laboratory scale to pilot production. Parallel, a molecular biotechnology project (cloning and expression of the enzyme) was initiated, aiming at the production of a highly concentrated and purer biocatalyst. Such an improved enzyme preparation was expected to greatly facilitate the downstream processing. By using a genetically engineered production organism the biocatalyst production should also become more cost-efficient due to higher fermentation yields of the desired enzymatic activity. Shortly after the pilot campaign, the enzyme responsible for the desired catalytic activity on ***S*-2** could be isolated, and the gene encoding it was cloned. A much purer and more active enzyme preparation was then produced[35] by transforming a suitable host cell with the DNA sequence encoding the esterase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.[36] The enzyme preparation thereby obtained was successfully tested in laboratory-scale experiments, where the removal of the biocatalyst before workup was not necessary. The final enzymatic process to be used in future production is thereby expected to be more economical and superior to the pilot-

(35) An enantiomeric ratio of $E > 200$ together with a significant higher specific activity was found (>80 times the activity of lot A). This allowed such a low biocatalyst dosage, that no emulsions formed during workup.

(36) Østergaard, P. R.; Mailand Hjort, C.; Deussen, H.-J.; Zundel, M.; Ebdrup, S.; Christensen, S.; Patkar, S. PCT-Appl. WO 02/12472.

plant process described in this article. Table 6 compares the major differences which have an economic impact of the initial laboratory procedure (first laboratory scale-up), the pilot-plant process described in this article, and a potential future production process, Furthermore, a laboratory procedure has been developed for the racemization of the unreacted ***R*-2**, which would allow the recycling of the unwanted enantiomer in a future production.[37]

Summary

Enzymatic activity for the enantioselective hydrolysis of ***S*-2** was identified by screening of over 80 hydrolytic enzymes. It was found that almost all enzyme preparations derived from *Aspergillus* fungi were highly enantioselective ($E > 200$). However, the activity of the enzyme preparations had to be improved for the desired catalytic activity to facilitate a good process. Increased enzymatic activity per volume culture broth of the enzyme of interest was obtained by optimizing the parameters of the fermentation of *Aspergillus oryzae*. Three pilot-scale fermentation batches were produced. A scalable chemoenzymatic process was developed for the enantioselective hydrolysis of ***S*-2** using these hydrolytic enzyme mixtures. A major process hurdle, the formation of emulsions during workup caused by the biocatalyst, could be circumvented by a simple precipitation procedure. The whole process was optimized and scaled up to pilot scale (43.9-kg scale). The isolated yields (44−48%), chemical purities (97.7−99.3%), and enantiomeric purities (98.4−99.6% ee) of the product ***S*-1** obtained from the pilot trials were excellent and consistent with the previous experiments. The only drawback was the occurrence of unexpected slow phase separations not seen previously during extraction. Nevertheless, the pilot trials were still technically feasible.

(37) ***R*-2** can be racemized with sodium ethoxide in ethanol or sodium hydride in THF.

Experimental Section

Experimental details about the enzyme screening results, the analytical methods, the chemoenzymatic process, and the *Aspergillus oryzae* fermentation have been published earlier.[9,10]

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