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Development of a Chemoenzymatic Manufacturing Process for Pregabalin

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Abstract:

A new manufacturing process for (*S*)-3-(aminomethyl)-5-methylhexanoic acid (Pregabalin), the active ingredient in Lyrica, has been developed. Using Lipolase, a commercially available lipase, *rac*-2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (1) can be resolved to form 2-carboxyethyl-3-cyano-5-methylhexanoic acid (2). A heat-promoted decarboxylation of 2 efficiently generates (*S*)-3-cyano-5-methylhexanoic acid ethyl ester (3), a known precursor of Pregabalin. This new route dramatically improved process efficiency compared to the first-generation process by setting the stereocenter early in the synthesis and enabling the facile racemization and reuse of (*R*)-1. The chemoenzymatic process also reduced organic solvent usage resulting in a mostly aqueous process. Compared to the first-generation manufacturing process, the new process resulted in higher yields of pregabalin (40–45% after one recycle of (*R*)-1), and substantial reductions of waste streams corresponding to a 5-fold decrease in the *E* factor from 86 to 17.

1. Introduction

(*S*)-(+)-3-aminomethyl-5-methylhexanoic acid (Pregabalin) is a lipophilic GABA (γ-aminobutyric acid) analogue that was developed for the treatment of several central nervous system disorders including epilepsy, neuropathic pain, anxiety and social phobia.[1,2] During the initial process development for pregabalin several routes were examined in detail.[3] The first generation manufacturing process (Scheme 1) was selected on the basis of overall yield, cost and high process throughput and executed as a racemic synthesis followed by resolution with (*S*)-(+)-mandelic acid. This route began with the Knoevenagel condensation of isovaleraldehyde and diethyl malonate, followed by cyanation to give the key intermediate **1**, which was established as the regulatory starting material. Compound **1** was then converted to racemic 3-aminomethyl-5-methylhexanoic acid in a three-step sequence that included a hydrolysis, a reduction, and a decarboxylation, all performed in one pot with a single isolation step. The crude racemic Pregabalin was then resolved using (*S*)-(+)-mandelic acid in a three-step crystallization process.

Although this route is cost-effective, there are possibilities for improvement in two areas: the use of an early as opposed to a late-stage resolution and the introduction of a recycling step to avoid discarding the undesired enantiomer. Several new routes have been under development.[4,5] The use of asymmetric hydrogenation as the key step for the reduction of 3-cyano-5-methylhex-3-enoic acid,[4] is an elegant potential manufacturing process but is surpassed in terms of cost effectiveness and environmental performance by the enzymatic route described in this paper. The use of enzymes, in particular hydrolases for the synthesis of chiral compounds has been extensively reviewed, and their application at large scale has also been reported.[6]

The route development described herein aimed at using an existing, relatively inexpensive racemic precursor **1** and introducing a hydrolase to generate an enantiopure precursor **2**. Conceptually, the generation of compound **2** from racemic precursor **1** involves the hydrolysis of one diastereotopic carboxyethyl group, a desymmetrization of the prochiral C-2 center. The desired outcome is to find an enzyme that will only perform such a reaction on a single enantiomer of racemic **1** (kinetic resolution), thus generating one (or two) diastereomers from a single enantiomer at the C-3 chiral center, leaving behind the *R* enantiomer of **1**. Thus, the diastereoselectivity in the desymmetrization reaction *per se* is not as important as the enantioselectivity of the kinetic resolution, as the chirality at the C-2 center is lost while converting **2** to Pregabalin. The initial plan also included the development of a procedure to recycle unused *R*-**1** enantiomer as well as new chemistry to convert **2** to Pregabalin (Scheme 2).

2. Results and Discussion

Development of a chemoenzymatic process to pregabalin involved four main steps: (1) screening to identify a suitable enzyme, (2) performing optimization of the enzymatic reaction to maximize throughput and minimize enzyme loading, (3)

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(5) Burns, M. P.; Weaver, J. K.; Wong, J. W. PCT Int. Appl. WO 2005100580(A1), 2005.

(6) (a) Drauz, K.; Waldmann, H. *Enzyme Catalysis in Organic Synthesis*, Wiley:Weinheim, 2002. (b) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*, 2nd ed.; Wiley:Weinheim, 2006. (c) Bornscheuer, U. T. Kazlauskas, R. J. *Hydrolases in Organic Synthesis*, 2nd ed.; Wiley:Weinheim, 2006..

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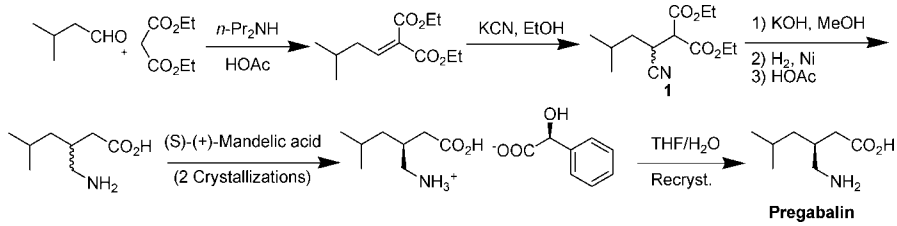
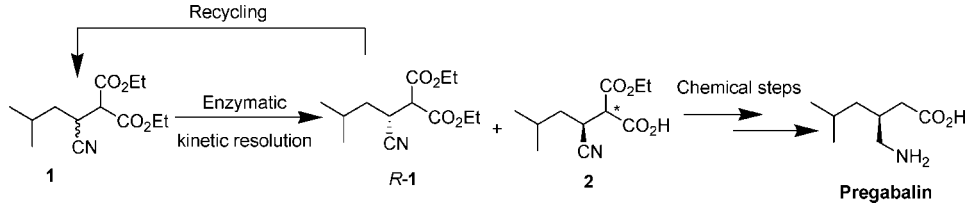
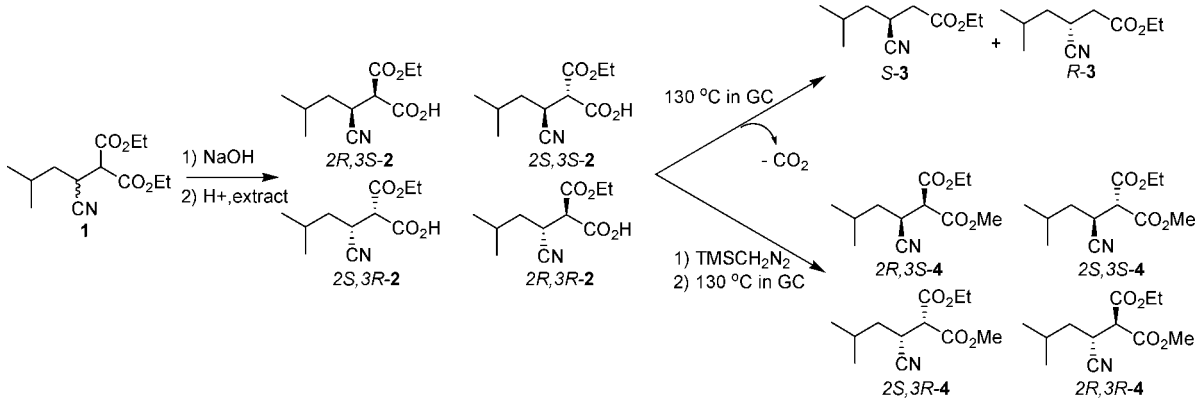
Scheme 1. **First generation Pregabalin manufacturing process**



Scheme 2. **Proposed route development**



Scheme 3. **Generation of standards 2, 3 and 4 for GC method development**



exploring a chemical pathway that could preserve the enantiopurity of the material already obtained and lead to Pregabalin, and (4) developing a procedure for the racemization of (*R*)-**1**. The introduction of a new synthetic intermediate **2** opens several possibilities in terms of developing a new process and also introduces a constraint that was absent in the first-generation manufacturing route in that the postresolution steps should be performed under conditions that will not epimerize the desired chiral center. The development of analytical methods for the study of this complex enzymatic hydrolysis reaction was crucial before any enzyme screening could be performed. The use of chiral capillary GC made a complete analysis of this reaction possible. The chemical generation of a racemic standard of compound **2** by a limited hydrolysis of racemic **1** at one of the carboxyethyl groups, gave rise to four diastereomers of **2** (see Scheme 3). When injected in the GC, the mixture of free carboxylic acids was only seen as two peaks. The analysis of these peaks by GC−MS revealed their identity as the enantiomers of 3-cyano-5-methylhexanoic acid ethyl ester **3**, due to the decarboxylation of **2** during GC analysis. This chiral GC method was used to determine the percent enantiomeric excess (% ee) of **3**, a value used as an indirect measure of the intrinsic enantioselectivity of the resolution. A derivatization method was also developed, which allowed the analysis of all four diastereomers of **2** as their methyl ethyl esters **4**, and enabled the determination of the true diastereoselectivity and enantioselectivity with a single analysis.

The apparently facile thermal decarboxylation of **2** during GC analysis suggested that thermal conditions could be used to carry out the conversion of **2** to **3** (see section 2.3).

2.1. Enzyme Screening. A screen of commercially available hydrolases was carried out in 96-well format to identify a suitable enzyme for the kinetic resolution of **1**.[7] Initial screening at a substrate loading of 5% (v/v) revealed many enzymes that catalyzed the hydrolysis of **1**, although only a few of these demonstrated enantioselectivity (after measuring enantiomeric ratios, *E* value[8]). (see Table 1).

Seven enzymes showed reasonable to good enantioselectivity ($E > 35$). The lipase from *Thermomyces lanuginosus*, commercially available as Lipolase, was the best in terms of enantioselectivity and activity. *Rhizopus delemar* and *Rhizopus niveus* lipases were also highly enantioselective, but both showed lower activity relative to Lipolase, based on reaction rates with equivalent amounts of enzyme. The less selective *Pseudomonas* sp. and *Mucor miehei* lipases, and *Mucor miehei* esterase were not evaluated further.

Lipolase was selected for process development based on its high enantioselectivity and activity for the hydrolysis of **1**. Although unimportant for the synthesis of Pregabalin, the enzyme was also highly diastereoselective (>99.5% *de* observed

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(8) Chen, C. S.; Fujimoto, Y.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1982**, *104*, 7294–99.

Table 1. **Enzyme hits found in the screening**

enzyme name	*E* value	selectivity	supplier
Thermomyces lanuginosus lipase (Lipolase)	>200	*S*	Novozymes
Rhizopus delemar lipase	>200	*S*	Amano
Rhizopus niveus lipase	66	*S*	Amano
Mucor miehei esterase	52	*S*	Sigma
Pseudomonas sp. Lipase	51	*S*	Sigma
Mucor miehei lipase	41	*S*	Novozymes
Rhizopus oryzae lipase	35	*S*	Amano
Candida antarctica lipase-A	5	*S*	Biocatalytics
Candida antarctica lipase-B	3	*S*	Biocatalytics
pig liver esterase	2	*S*	Biocatalytics
enteropeptidase	2	*S*	Sigma
porcine kidney acylase	2	*S*	Sigma
cholesterol esterase	2	*S*	Biocatalytics
Streptomyces griseus protease	20	*R*	Sigma
Streptomyces sp. protease	11	*R*	Biocatalytics

Table 2. **Initial optimization for Lipolase resolution of 1**[a]

parameter	evaluated	set value
pH	6–9	8.0
temperature	4–40 °C	25 °C
ionic strength	0–500 mM	10 mM
enzyme loading	1–20% (v/v)	1% (v/v)
buffer	Tris, phosphate	phosphate
cosolvent	0–30%	0%
substrate concn	0–1 M	0.8 M
substrate add'n	single and fed batch	single

[a] Experiments were performed at 0.8 M concentrations of **1,** and 16–20 h reaction samples obtained (30–50% conversions).

when the product **2** was converted to the methyl ethyl esters **4**). The commercial availability of Lipolase and its low cost provided further advantages for its potential use in a manufacturing process. Lipolase is produced by Novozymes using a submerged fermentation of a genetically modified *Aspergillus oryzae* strain.[9] The protein has a molecular mass of 30 kDa (269 amino acids) and belongs to the α/β hydrolase fold family of enzymes, which are subject to interfacial activation.

2.2. Reaction Optimization with *Thermomyces lanuginosus* Lipase. During the early stages of optimization, an initial goal was set to start with a 1 M concentration of **1** in the reaction and to perform the reaction in less than 24 h. This goal was achieved relatively easily, mainly because of the high catalytic activity of Lipolase in the hydrolysis reaction. A summary of the set process values is presented in Table 2. It should be noted that there were different optimal operating parameters for different substrate concentrations and the set values chosen initially corresponded to a reaction carried out at 0.8 M substrate concentration in 14–16 h. The system displayed good tolerance to **1** up to a concentration of 1 M, after which resulted in incomplete reactions (<40% conversion).

A closer look at the pH and temperature effects in a reaction at 1.5 M substrate concentration revealed that conditions that increased the reaction rate also tended to inactivate the enzyme. In Table 3, at 3.5 h reaction time, pH 8.0 might seem better than pH 7.0. However, the conversion values obtained after 18 h revealed that a lower conversion is observed at pH 8.0. A similar observation was made for the reaction at pH 7.0 at two different temperatures. Although higher temperature and pH do display higher initial rates, they also deactivate the enzyme faster, leading to lower observed conversions after 18 h.

(9) Esper, B.; Huge-Jensen, I. B. EP0305216B1, 1995.

Table 3. **Impact of pH and temperature on conversion**[a]

	conversions			
time (h)\pH	6.6	7.0	7.0[b]	8.0
3.5	11	13	20	14
18	33	37	36	28

[a] Experiments were performed at 1.5 M concentrations of **1** and 3% lipolase (v/v) in potassium phosphate buffer at 0.1 M concentration at 25 °C. [b] Reaction run at 30 °C

In order to further examine potential sources for the enzyme inactivation at higher substrate concentrations, the mode of addition of **1** was examined. This study revealed that fed batch additions of **1** gave almost equal conversions compared to a single addition (Figure 1a), clearly indicating that there were no signs of strong substrate inhibition. To test for product inhibition, addition of the sodium salt of acid **2** to the reaction mixture at t_0 was examined. The study revealed that, at 0.1 M concentrations, product **2** had a significant inhibitory effect on the rate of the reaction (Figure 1b). It then became clear that the main barrier to increasing throughput of this step was the product inhibition.

A review of the literature examining potential approaches avoiding product inhibition suggested the addition of agents that could form a complex or salt with carboxylic acid **2**, thus minimizing its ability to deactivate the enzyme.[10] The evaluation of ion exchange resins such as Dowex and AG type at concentrations up to 5% (w/v) did not suppress the inhibition to any extent. The use of bases other than sodium hydroxide that could supply a different counterion for **2** was also tested.[11] Ammonium hydroxide, calcium hydroxide, and several amines (*tert*-butylamine, di-*n*-propylamine, etc.) all yielded equal or lower rates with no suppression of inhibition.

The addition of divalent ion species to the reaction (see Table 4), proved to be the best solution. Calcium and zinc ions significantly suppressed the inhibition, possibly by forming a complex that remained suspended in the emulsion and prevented the inactivation of the Lipolase in the reaction mixture.

The effect of calcium acetate in the reaction medium at higher concentrations of **1** was then examined in more detail. Surprisingly, the enzymatic reaction proceeded without a

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(11) Nomoto, F.; Hirayama, Y.; Ikunaka, M, J.; Inoue, T.; Otsuka, K. *Tetrahedron: Asymmetry* **2003**, *14*, 1871–1877.

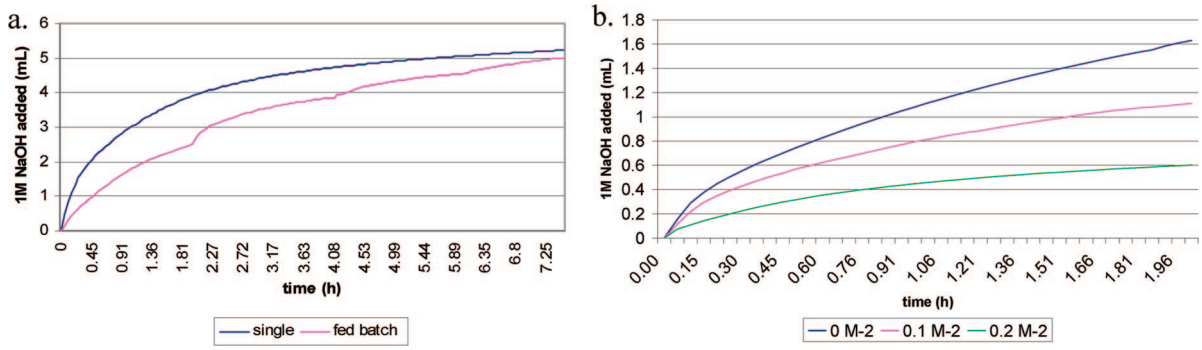


Figure 1. **Substrate and product inhibition test. (a) Experiments performed at 2 M concentrations of 1, in a single *vs* four 0.5 M additions. (b) Experiments performed by adding, at t_0, compound 2, then setting pH to 8.0, and adding enzyme and substrate at 1 M concentration.**

Table 4. **Counterion buffer effect[a]**

time (h)	K/PO_4	NaOAc	KOAc	NH_4OAc	$Ca(OAc)_2$	$CaCl_2$[b]	$Mg(OAc)_2$	$Mn(OAc)_2$	$Zn(OAc)_2$	$Ba(OAc)_2$
2	15	15	15	12	18	11	10	13	15	15
3	18	20	19	16	22.5	18	17	15	16	19
4	20	21	21	20	27	23	19	16	20	21
5	21	23	23	20	31	27	20	18	23	24
22	30	–	–	–	44	46	33	38	42	34

[a] Experiments were performed at 1.5 M concentrations of **1** and 3% Lipolase (v/v) in different buffer salts at 0.1 M concentration and pH 7.0. [b] $CaCl_2$ in 10 mM potassium phosphate buffer.

Table 5. **Effect of substrate 1 and Lipolase concentration[a]**

entry	[**1**]/M	$[Ca(OAc)_2]$/M	[Lipolase]/%	conversion/%
1	1.5	0.1	3	47.5
2	2	0.12	4	43.2
3	2.5	0.15	5	43.1
4	3	0.17	6	42
5	3	0.15	1.2	20
6	3	0.15	2.4	29.5
7	3	0.15	6	41.3
8	3	0.15	8	45
9	3	0.15	12	47.5

[a] Conversion values correspond to samples taken at 25 h.

problem at substrate loads as high as 3 M or 765 g/L; with conversion values ranging from 42 to 48% after 24 h (see Table 5, entries 1–4). Since the concentration of calcium acetate used in those experiments did not exceed 170 mM, which is well below a stoichiometric ratio between the carboxylate of **2** and Ca^{2+}, a more complex mechanism that probably involves enzyme stabilization as well as complexation of product might be taking place.

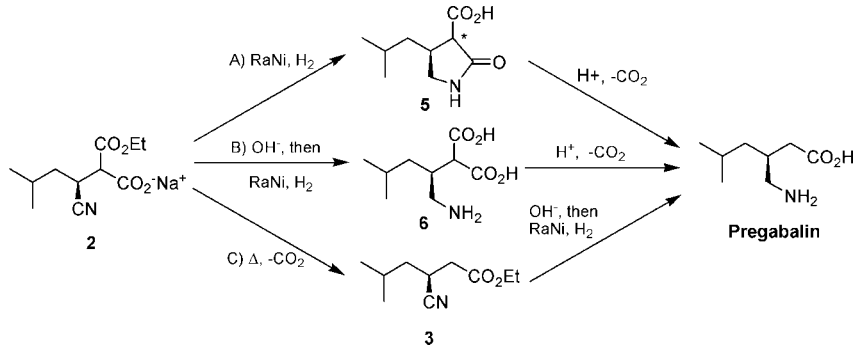
By tuning the amount of lipolase used (see Table 5, entries 5–9), the enzymatic reaction could be performed in the 24 h window initially set as desirable. A total turnover number close to 10^5 was observed. An added benefit that came with the suppression of product inhibition, mainly as a result of increasing the substrate concentration to 3 M (765 g **1**/L reaction), was a dramatic improvement in the phase separation at the end of enzymatic reaction. Complete phase splitting was achieved in just a few minutes (*vs* several hours at 1 M **1**) mainly due to two changes in the reaction solution properties. First, the proportion of organic to aqueous layers increased greatly at 3 M compared to 1 M substrate **1** (3:1 *vs* 1:3 respectively, at t_0); second, the density of the aqueous solution at the end of the reaction also increased as a result of the larger amount of carboxylate salt of **2** present, thus improving the phase separation. The optimized conditions were tested for robustness in multiple runs (>20) at 10 kg scale. Reactors with standard agitation range were used during scale-up above 10 kg with no special modifications made. Three pilot runs at 900 kg (1600 L reactor) scale as well as manufacturing trials at 3.5*t* (8000 L reactor) demonstrated the consistently high performance and scalability of this enzymatic reaction.

2.3. Subsequent Chemical Steps to Pregabalin. The chemical transformation of the enantiopure acid **2** into Pregabalin could only be performed under neutral or basic conditions, mainly because of the low stability of **2** under acidic conditions. Three pathways (Scheme 4) were then considered.

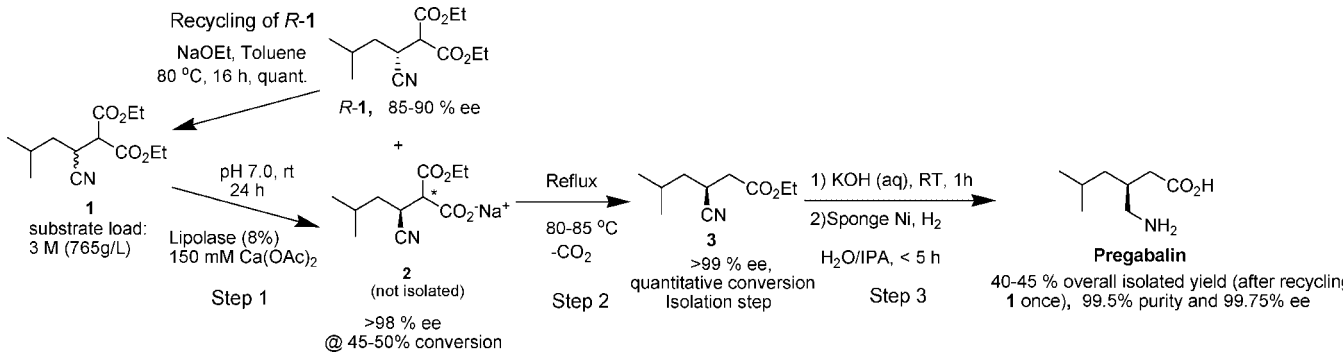
Pathway A employed a reductive cyclization[12] to form 3-carboxy-4-alkyl-pyrrolidin-2-one (**5**), which could then be converted to Pregabalin under acidic conditions. Pathway B employed a hydrolysis/reduction/decarboxylation analogous to the one used in the first-generation route (Scheme 1).[3] Pathway C involved a heat-mediated decarboxylation to intermediate **3**, followed by a hydrolysis-reduction sequence to yield pregabalin. The pathway A hydrogenation of **2** was performed in predominantly aqueous medium at neutral pH, with catalytic amounts of Raney nickel, and afforded **5** in >95% isolated yield. The reduction could be performed at relatively high substrate loads (0.5–1.0 M), and the lactam hydrolysis−decarboxylation step associated with this path (using 2.5 equiv of 4 N HCl and catalytic HOAc at 120 °C) yielded 70–80% crude Pregabalin over the two steps. The main drawbacks found with this path came about when testing the reduction reactions at substrate concentrations ranging from 1.0 to 2.5 M. Poisoning of Raney nickel, due to the presence of enzyme in the solution, gave rise to incomplete conversion. To overcome this problem, Raney

(12) Koelsh, C. F.; Stratton, C. H. *J. Am. Chem. Soc.* **1944**, *66*, 1883–1884.

Scheme 4. **Proposed chemical transformations of 2 to Pregabalin**



Scheme 5. **Optimized chemoenzymatic Pregabalin synthesis**



nickel loads as high as 20 mol % were tested with limited success, seriously hurting the chances to scale up this path above 1 kg scale.

Pathway B utilized conditions from the first generation manufacturing route[3] where **1** is used as the starting material instead of **2**. Less exploratory work was done on this pathway, mainly due to the higher risk of epimerization of the C3 center under highly alkaline conditions as well as the difficult isolation of **6**, which implied carrying enzyme (as well as enzyme byproducts) to the API isolation step. The poisoning of the Raney nickel by enzyme, previously observed in pathway A, also made this an undesirable option.

Pathway C was first explored using Krapcho decarboxylation conditions on free acid **2** (135 °C, DMSO−NaCl), yielding a mixture of compound **3** and other uncharacterized decomposition products. The reaction proceeded very slowly and resulted in incomplete conversions. To minimize the exposure of **2** to acidic conditions, which were required for the preparation of free acid **2**, a decarboxylation under neutral conditions was attempted. The process consisted of simply heating the aqueous solution from the enzymatic reaction (after phase separation) to reflux (70–95 °C). Under these conditions, a rapid chemical decarboxylation of **2** into **3** took place in less than 5 h, without any racemization and in the presence of enzyme in solution. This path turned out to be the best possible solution, as it generated a water insoluble oil (**3**), leaving behind in the aqueous layer potential impurities including enzyme, buffer, and calcium salts that could affect the hydrogenation step and the purity of the pregabalin at the final step. CO_2 evolution was not an issue during scale-up, and a standard vent was used.

2.4. Recycling of the Remaining Ester (*R*)-1. Once an efficient enzymatic kinetic resolution was developed, recycling of the remaining enantiomer (*R*)-**1** was explored. The first attempt to use sodium ethoxide in ethanol at room temperature resulted in partial racemization (only 5−10% in 16 h). After evaluating the reaction at higher temperatures, a significant effect on the rate of racemization was found (results not shown). When the racemization was carried out at 80 °C more than 98% racemization was achieved in 8–16 h. Current efforts involving the use of a continuously operated recycling system have revealed the potential to run a higher-yielding dynamic resolution process. Such improvement might be implemented in the future.

2.5. Optimized Route to Pregabalin. Having developed the high-throughput enzymatic resolution and decarboxylation steps, the end game towards Pregabalin was carried out with minor modifications of published procedures,[3] *i.e.* KOH hydrolysis of **3** followed by hydrogenation catalyzed by Raney nickel to obtain the enantiopure Pregabalin API (Scheme 5). The one-pot hydrolysis/reduction reaction occurred without any racemization, and faster rates as well as higher yields and purities compared to those from the first-generation process were obtained: 40–45% overall isolated yield for Pregabalin (after recycling **1** once) with 99.5% purity and 99.75% ee.

The efficiency of the route can be appreciated by examining the ratio of the kilograms of total waste to kilograms of pregabalin product (*E* factor).[13] An *E* factor of 17 was calculated for the chemoenzymatic route relative to 86 for the first-generation (classical resolution) route. This index provides a simple comparison between the two routes in terms of the environmental impact associated to each of them. The examination of the total reagent usage in the two processes (Table 6) clearly shows that the new route utilizes 5 times less input of chemicals and 8 times less input of solvents as compared to

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Table 6. **Inputs for 1000 kg of Pregabalin via first-generation and new route**

inputs	first-generation route (kg)	new route (kg)
1	6212	4798
enzyme	0	574
(*S*)-mandelic acid	1135	0
Raney nickel	531	79.5
solvents	50042	6230
total	57920	11681.5

the first-generation route. Moreover, in the optimized process every chemical reaction is run in water with minimal solvents used for workup. Some of the process water can be sent directly to the wastewater treatment plant, and the solvent from the hydrolysis/decarboxylation process is recovered. Further improvement from pilot-plant and production scale runs have been demonstrated and will be implemented in the future.

3. Summary

A scalable manufacturing process for Pregabalin has been developed. The process uses a commercially available lipase from *Thermomyces lanuginosus* (Lipolase) to prepare the key enantiopure intermediate **2**. The enzymatic process features high yields and an unprecedentedly high substrate load, and is performed early in the synthetic scheme. Furthermore, removal of the undesired ester involving a rapid phase splitting and its facile racemization enabled the recycling step of unreacted starting material **1**, a step not possible in the first-generation route. The rapid decarboxylation of **2** yielding a water-insoluble intermediate **3**, followed by a simple hydrolysis/reduction sequence afforded Pregabalin in high yield and purity. This newly developed route to Pregabalin makes use of an existing key intermediate (**1**), and utilizes an API isolation sequence (after isolation of **3**) very similar to the one already used in the first-generation route, making it a readily implemented second-generation route. In addition to implementation of the biotransformation step, the new route also demonstrates the application of green chemistry by using water in all three reaction steps, by eliminating two isolations, and by decreasing the amount of waste generated. The improvements in the *E* factor from 86 to 17 and a 5-fold reduction in total chemical usage input relative to that from the first-generation route make this a superior and more environmentally benign approach towards Pregabalin.

4. Experimental Section

Materials. All enzymes included in the screening plate (see Supporting Information) were obtained from commercial enzyme suppliers including Amano (Nagoya, Japan), Roche (Basel, Switzerland), Novozymes (Franklinton, NC), Altus Biologics Inc. (Cambridge, MA), Biocatalytics (Pasadena, CA), Toyobo (Osaka, Japan), Sigma and Fluka (see ref 6 for details on preparation of screening plate, including specific enzyme sources for each enzyme, as well as a detailed description of the screening methodology). Enzyme screening was performed in an Eppendorf thermomixer-R (VWR). HPLC analysis was performed on an Agilent 1100 HPLC with 96-well autosampler and using HPLC columns obtained from Phenomenex (Torrance, CA). GC analysis was performed on an Agilent 6890N network GC system equipped with an FID detector, a 7683 series split/splitless capillary injector, and a PAL 96-well autosampler and using columns obtained from Astec, Inc. (Whippany, NJ). Commercially available Lipolase 100L-EX was purchased from Novozymes and was usually shipped with a declared activity of 100,000 lipase U/g of solution. Raney nickel, Dowex resins, and other chemicals were purchased from Aldrich-Sigma. AG resins were purchased from Biorad.

Analytical Methods. GC method for the analysis of compounds **1**, **2**, **3**, and **4**: G-TA (30 m × 0.25 mm), constant oven temperature (135 °C), helium as carrier gas, FID as a detector. 50 μL of the Lipolase reaction were mixed with 1 mL of MTBE and 50 μL of 1 M HCl, and the MTBE phase was diluted 10 times in 1 mL of dry MTBE and submitted for a chiral GC directly or derivatized by adding 40 μL of dry methanol and 40 μL of TMS diazomethane prior to injecting. Nonchiral RP-HPLC method for the analysis of compounds **1**, **2**, **3**, and **4**: Luna 3 μ C18(2), 4.6 mm × 150 mm, 35 °C, injection volume 10 μL, Solvent A: 20 mM $NaHPO_4$, pH 2.0, Solvent B: acetonitrile, gradient 10–90% B in 10 min, then 90% B for 3 min, at 1.2 mL/min and detection at 210 nm using a refractive index detector. Samples were dissolved in acetonitrile or methanol. The optical purity of Pregabalin was analyzed via derivatization with Marfey's reagent[14] and analyzed on a C_{18} column and the method described above. A chiral HPLC method was used to measure the % ee value of **5**, after esterification with EtOH under catalytic dry HCl in dioxane at 70 °C. The obtained lactam ester was analyzed by Chiralpak AD-H (4.6 mm × 250 mm) using hexane/EtOH (95:5) as mobile phase, 1.0 mL/min and detection at 210 nm.

Procedure for Enzyme Screening. The resolution of racemic ester **1** was carried out as follows. A 96-well screening plate prepared in-house[7] was thawed for 5 min. Then, 85 μL of potassium phosphate buffer (0.1 M, pH 7.2) were dispensed into the wells using a multichannel pipet. 5 μL of neat substrate was then added to each well via a multichannel pipet, and the 96 reactions were incubated at 30 °C and 750 rpm. The reactions were quenched after 24 h, by transferring the reaction mixture into a new 2-mL deep 96-well plate containing 1 mL of EtOAc and 100 μL of 1 N HCl. After mixing, the 96-well plate was centrifuged, and 100 μL of the organic supernatant were transferred from each well into another 96-well plate, then sealed using a penetrable mat cover and transferred to a GC system for chiral analysis.

Preparation of (*S*)-2-Carboxyethyl-3-cyano-5-methylhexanoic Acid (2). In a suitable reactor was charged a premixed solution of deionized water (2103 mL) and $Ca(OAc)_2$ hemihydrate (282.5g). Lipolase commercial solution (800 g, 32 g actual protein) was then charged, and the resulting solution was stirred at 400 rpm. Cyano diester **1** (10.0 kg) was charged as a liquid in one portion, and the autotitrator started maintaining the pH of the reaction at

(14) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.

7.0 using 30% NaOH (w/w) solution. After 24 h, a GC sample was taken (45–48% conversion), and titrator and agitation were stopped. Layers were settled for 15–30 min, and the upper layer separated. Toluene (1.0 L) was then charged to the aqueous solution, and the resulting mixture stirred for 10 min. Layers were settled for 15–30 min, and the upper (organic) layer separated and was combined with the (*R*)-**1** solution previously separated. The aqueous layer was then taken forward to the next step without any further workup or isolation. For spectral characterization, a *tert*-butylamine salt of **2** was prepared: 1 L of an aqueous solution of **2** (~1 mol) was acidified to pH 3.0 by adding concentrated HCl, and the free acid was extracted with 0.5 vol. of MTBE (three times). To the combined MTBE free acid solution was charged *tert*-butylamine (1 equiv) as a solution with 0.1 vol. of isopropanol. The solid was collected (>95% recovery, >95% pure) and recrystallized from 1 vol. of isopropanol and dried in a vacuum oven using house vacuum at 50 °C. Mp 135.4 °C, MS (acid) *m*/*z* $[M + H]^+$ 227, ^{1}H NMR (for *tert*-butylamine salt 300 MHz, D_2O): δ 0.91 (dd, 6H), 1.28 (t, 3H), 1.37 (s, 9H) 1.60 (m, 1H), 1.78 (m, 1H), 3.29 (m, 1H), 3.50 (d, 1H), 4.22 (q, 2H), 4.22 (s, 3H). ^{13}C NMR (75 ppm, D_2O) δ 13.73, 20.88, 22.61, 26.20, 27.11, 30.20, 38.65, 52.39, 57.25, 62.99, 122.90, 171.02, 172.54.

Preparation of (*S*)-3-Cyano-5-methylhexanoic Acid Ethyl Ester (3). An aqueous solution from the Lipolase reaction (2 L, ~3 mol) was charged in a suitable reactor and heated to 85 °C for 3 h while stirring under a nitrogen atmosphere. The mixture was then cooled to 50 °C, and layers were settled for 15–30 min. The organic layer containing the neat crude compound **3** was then transferred into the next step (KOH hydrolysis). The spectral data on this compound (MS, ^{1}H NMR, ^{13}C NMR) were in agreement with those reported previously.[3]

Preparation of 3-Carboxy-4-alkyl-pyrrolidin-2-one (5). A suitable hydrogenation vessel was charged with an aqueous solution of **2** (100 mL, ~0.15 mol), followed by 21.5 mL of Raney nickel water solution (50%) and the mixture hydrogenated at 50 psi for 20 h. After reaction completion, the catalyst was filtered. The pH of the remaining solution was adjusted to 3.0 using 37% HCl and the solution extracted three times with 0.5 vol of EtOAc. The combined organic layers were concentrated *in vacuo* to afford 26.5 g of compound **5** as an oily residue, in 40–42% isolated yield. Compound **5** was analyzed using the chiral HPLC method described above and found to be present in >97% ee. MS *m*/*z* $[M + H]^+$ 186.1130, ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.87 (d, 6H), 1.33 (m, 1H), 1.51 (m, 2H) 2.88 (m, 1H), 2.99 (t, 1H), 3.06 (d, 1H), 3.54 (t, 1H), 7.64 (s, 1H), 9.80 (b, 1H); ^{13}C NMR (75 ppm, $CDCl_3$) δ 175.67, 172.23, 54.09, 47.62, 43.69, 37.22, 26.31, 23.34, 22.54.

Preparation of (*S*)-3-(Aminomethyl)-5-methylhexanoic Acid (Pregabalin) from 3. The crude oil **3** was converted to crude Pregabalin following previously published procedures with minor modifications. The spectral data on this compound (MS, ^{1}H NMR, ^{13}C NMR) were in agreement with the previous report.[3]

Preparation of (*S*)-3-(Aminomethyl)-5-methylhexanoic Acid from 5. A suitable vessel was charged with 50 g (~80% purity) of **5** and a solution of 41 mL of concentrated HCl and 41 mL of water. Then 4 mL of glacial HOAc were charged, and the resulting slurry was refluxed for ~36−48 h at 80 °C. The temperature of the reaction was then increased to 110 °C and reaction continued for another 6 h. The water and excess HCl were evaporated to afford an oily residue. The residue was washed with MTBE (15 mL × 2), and water (54.5 mL) was then added, and the mixture was stirred until a clear solution was formed. The pH of the solution was adjusted to 5.2–5.5 with 45% KOH, when a precipitate was formed. The mixture was heated up to 70 °C and then cooled down to 4 °C. After 10 h, crystalline Pregabalin (19.5 g) was filtered and washed with cold IPA (20 mL). To collect a second crop of crystals, the filtrate was concentrated to afford an oily residue, followed by addition of H_2O (22 mL) and ethanol (22 mL). The mixture was heated up to 70 °C and then cooled down to 4 °C. After 10 h, a second crop of crystalline Pregabalin (8.0 g) was filtered and washed with IPA (15 mL). The combined crystals were dried in a vacuum oven at 45 °C for 24 h to afford enantiopure Pregabalin (in 80% yield and >99.5% ee). The spectral data on this compound (MS, ^{1}H NMR, ^{13}C NMR) were in agreement with the previous report.[3]

Recycling of *rac*-2-Carboxyethyl-3-cyano-5-methylhexanoic Acid Ethyl Ester (1) from *R*-1. A suitable vessel was charged with 1 kg of (*R*)-cyanodiester **1** from step 1 as a toluene solution, followed by 1.42 kg of 21 wt % sodium ethoxide solution in ethanol (1.1 equiv). The mixture was then heated to 80 °C for 8–16 h. After reaction completion, the mixture was cooled down to 4 °C and neutralized by adding 0.27 L of glacial acetic acid. The slurry was filtered, and the filtrate was evaporated to afford racemic ester **1**. The material was then distilled following literature procedure[3] to afford *rac*-**1** in >95% isolated yield. The spectral data on this compound (MS, ^{1}H NMR, ^{13}C NMR) were in agreement with the previous report.[3]

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