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Enzyme-Catalyzed Hydrolysis of Bicycloheptane and Cyclobutene Diesters to Monoesters

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Supporting Information

ABSTRACT: Diacid formation is a major problem in the conventional chemical hydrolysis of a diester to a monoester. Enzyme-catalyzed hydrolysis of dimethyl bicyclo[2.2.1]heptane-1,4-dicarboxylate (**1**) by lipases from *Candida antarctica* and *Burkholderia cepacia* gave the corresponding monoester 4-(methoxycarbonyl)bicyclo[2.2.1]heptane-1-carboxylic acid (**2**) in excellent yields with negligible amounts of diacid **3**. About 100 kg of monoester **2** was prepared in 78% yield by hydrolysis of diester **1** with a commercially available lipase from *B. cepacia*. A more efficient process for the hydrolysis of **1** that give monoester **2** in 82% yield was subsequently developed using significantly lower amounts of the commercially available immobilized lipase B from *C. antarctica*. The commercially available immobilized lipase B from *C. antarctica* and porcine liver esterase were also efficient for the selective hydrolysis of dimethyl cyclobut-1-ene-1,2-dicarboxylate (**4**) to the corresponding monoester **5** in yields of 78% and 87%, respectively.

INTRODUCTION

A useful approach for desymmetrization of a symmetric diester is its selective hydrolysis to the corresponding monoester.[1,2] Diacid formation is a major problem in the conventional chemical hydrolysis of a diester. In the case of the diester dimethyl bicyclo[2.2.1]heptane-1,4-dicarboxylate (**1**),[1−7] significant levels of diacid were generated with potassium hydroxide,[1,4,6] sodium hydroxide,[5,7] or barium hydroxide[2] as the base even at <100% conversion (Scheme 1). The downstream isolation process for the separation of the remaining diester **1**, the desired monoester **2**, and the undesired diacid **3** required careful pH control and was tedious. Monoester **2** is a building block of many potential therapeutic candidates.[5−7] Enzymatic hydrolysis is a powerful tool for the selective hydrolysis of esters.[8,9] Hydrolysis and desymmetrization of diesters to monoesters by porcine liver esterase,[10−14] porcine pancreatic lipase,[11,12] lipase from *Psudomonas* species,[13] lipase from *Candida antarctica*,[12,14,15] and other lipases[16,17] have been reported extensively in the literature. We previously reported[4] preliminary results of our work on the synthesis and chemical and enzymatic hydrolysis of **1**. This paper describes detailed work on the selective enzyme-catalyzed hydrolysis of **1** and dimethyl cyclobut-1-ene-1,2-dicarboxylate (**4**) to their corresponding monoesters **2** and **5**, respectively.

RESULTS AND DISCUSSION

Hydrolysis of Dimethyl Bicyclo[2.2.1]heptane-1,4-dicarboxylate (1) to Monoester 2. *Enzyme Screening.* More than 100 hydrolytic enzymes were screened for the hydrolysis of **1**. The promising enzymes identified are listed in Table 1. Many enzymes showed good hydrolytic activities and gave monoester **2** as the major product. Most lipases that showed hydrolytic activity also had good selectivity for monoester **2** and produced undetectable or negligible amounts of diacid **3**. On the other hand, proteases that showed hydrolytic activity showed lower selectivity and produced higher levels of diacid **3** along with monoester **2**. Lipases from *C. antarctica*, *Candida rugosa*, and *Burkholderia cepacia* (previously known as *Pseudomonas cepacia*) showed the highest activities and selectivities. After a few small-scale experiments, the lipase from *B. cepacia* (Amano Lipase PS-30) was found to have the best combination of activity and selectivity. We had several kilograms of this lipase in our stock and dedicated our efforts to the quick development of a process for preparing monoester **2** on the larger scale required for the development of a prospective drug candidate.

Process Development Using the Lipase from B. cepacia, Lipase PS-30. The effects of cosolvent, temperature, and pH on Lipase PS-30-catalyzed hydrolysis of diester **1** were evaluated. Seven commonly used cosolvents (acetonitrile, isopropanol, *tert*-butanol, tetrahydrofuran, 2-methyltetrahydrofuran, dimethylformamide, and dimethyl sulfoxide) were evaluated at the 10% v/v level, but none of them showed any improvement; consequently, a cosolvent was not used for scale-up. The effects of temperature and the pH of the starting buffer were evaluated on a 1 mL scale with a substrate concentration of 20 g/L at various levels of enzyme loading. As expected, the reaction rate increased with increasing temperature. The reaction was faster when the starting buffer was at pH 7 compared with either pH 6 or pH 8. The pH dropped significantly to 4−5 in the small-scale reactions without any pH control.

The optimum conditions were developed by carrying out the reaction on a larger scale at constant pH and temperature. A constant pH of 7, a reaction temperature of 40 °C, a diester **1** input of 50 g/L, and a Lipase PS-30 concentration of 25 g/L

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Scheme 1. Hydrolysis of Dimethyl Bicyclo[2.2.1]heptane-1,4-dicarboxylate (1)

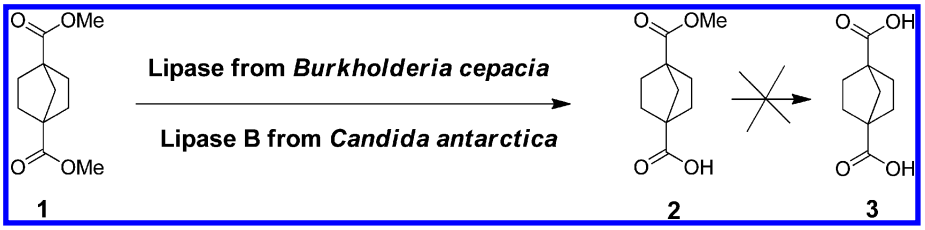

Table 1. Promising Enzymes for the Hydrolysis of Diester 1

			amounts (%) at the end of the reaction		
enzyme	source	supplier	diester **1**	monoester **2**	diacid **3**
Lipase PS-30	*Burkholderia cepacia*	Amano	3	97	<0.5
Lipase SL	*Burkholderia cepacia*	Meito Sangyo	3	97	<0.5
Lipase OF	*Candida rugosa*	Meito Sangyo	2	98	<0.5
Novozym 435	*Candida antarctica*	Novozymes	1	98	1
Lipase M-10	*Mucor javanicus*	Amano	64	36	<0.5
Lipase AY-30	*Candida rugosa*	Amano	68	32	<0.5
Protease P-6	*Aspergillus melleus*	Amano	67	27	6
Protease P4032	*Aspergillus oryzae*	Sigma	62	31	7
porcine liver esterase	porcine liver	Sigma	17	3	80

(2:1 substrate to enzyme ratio) were found to be optimal for the reaction.

Three hydrolysis batches were carried out under the optimum conditions using 251, 266, or 304 g of crude diester **1** in the batch. The diester **1** used in these experiments was crude, with potencies of about 87%, and contained considerable amounts of several unknown impurities originating from its synthesis. The enzyme tolerated the impurities, and the reaction reached 98% conversion in 24 h with negligible formation of diacid **3** in all cases. Most of the impurities were removed together with the remaining diester **1** during the extraction with MTBE at pH 8. The aqueous phase was then immediately acidified and extracted with MTBE to obtain monoester **2** with an average "as is" yield of 78% and corrected (for the potency of starting material) yield of 90% for the three batches. The success of the enzymatic hydrolysis step with crude diester **1** and the formation of pure monoester **2** in high yield and high purity showed that the process is robust and efficient.

Lipase from B. cepacia, Lipase PS-SD. After developing the process for scale-up with Lipase PS-30 from *B. cepacia*, we required a large supply of Lipase PS-30 to prepare large quantities of monoester **2**. However, we learned that Lipase PS-30 was no longer commercially available and was replaced by Lipase PS-SD obtained from the same source (*B. cepacia*) by its supplier, Amano. Though both Lipase PS-30 and Lipase PS-SD have the same activity (as per certificate of analysis from Amano) of 30 000 units/g for the hydrolysis of the standard substrate olive oil, Lipase PS-SD was found to have only one-third the activity of Lipase PS-30 for the hydrolysis of diester **1**. The difference in activities for the standard substrate (olive oil) and the specific compound **1** displayed by the two enzyme formulations from the same source highlights an important issue. The activity of the enzyme with the actual substrate rather than a general compound is the key criterion for its use in a biocatalytic synthetic step. Wells et al.[18] also suggested the activity of the enzyme versus the actual substrate as a key specification of enzymes for the synthesis of active pharmaceutical ingredients. Lipase PS-30 is a versatile enzyme, and there are numerous reports in the literature on its use in organic synthesis.[19−21] Since Lipase PS-30 is no longer commercially available, it would be interesting to see how the new formulation Lipase PS-SD would behave in organic synthesis examples reported in the past. The difference between the activities of Lipase PS-30 and Lipase PS-SD could not be due to possible differences in fillers commonly used in solid enzyme formulations since two common fillers (diatomaceous earth and dextrin) had no effect on their activities for the hydrolysis of diester **1**. Fortunately, the selectivity (monoester **2** to diacid **3** ratio) of Lipase PS-SD for the hydrolysis of diester **1** was high, similar to that of Lipase PS-30. We decided to quickly optimize and develop a process to scale-up the hydrolysis of **1** with Lipase PS-SD to supply monoester **2** in time for development studies, knowing fully that a large ratio of Lipase PS-SD to substrate would be required and that a better enzyme might be required in the long run.

Process Development Using Lipase PS-SD from B. cepacia. Again, eight commonly used cosolvents were evaluated, and as in the case of Lipase PS-30, there was no improvement; thus, a cosolvent was not used for further Lipase PS-SD process development. Lipase PS-SD-catalyzed hydrolysis of diester **1** was evaluated on a 1 mL scale with 20 mg of substrate and various enzyme concentrations at different temperatures for 24 h. The best temperature was found to be 50 °C. However, there was a sharp drop in activity above 50 °C. Hence, the temperature was maintained between 45 and 50 °C for optimum enzyme activity and was carefully controlled to avoid overheating. With 20 g/L substrate and 30 g/L Lipase PS-SD at 50 °C, the conversion reached 99.5% in 24 h and produced a negligible amount of diacid **3**.

Scale-Up of the Process Using Lipase PS-SD from B. cepacia. The optimum conditions for the Lipase PS-SD-catalyzed hydrolysis were developed, and the enzymatic hydrolysis was carried out on increasing scale. A constant pH of 7, a reaction temperature of 48° ± 2 °C, a diester **1** input of 50 g/L, and a Lipase PS-SD concentration of 75 g/L (2:3 substrate to enzyme ratio) were found to be optimal for the reaction.

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The hydrolysis was scaled up to a 405 g batch of crude diester **1** using 600 g of Lipase PS-SD at 50 g/L substrate input at 48 ± 2 °C. The conversion reached 97.7% at 25 h with negligible diacid **3** formation. After workup, 300 g of monoester **2** (79% "as is" yield, 90% yield after correction for the potency of starting diester) was obtained.

The Lipase PS-SD-catalyzed hydrolysis process was then scaled up to 80 kg of crude diester **1** input to obtain 58 kg of monoester **2** in 78% "as is" yield with high purity (AP > 97; diester **1**, AP < 0.5; diacid **3**, AP < 0.5). About 100 kg of total monoester **2** was produced by the Lipase PS-SD-catalyzed hydrolysis of diester **1** to support preparation of a drug candidate. The optimum substrate to enzyme ratio for Lipase PS-SD (2:3) is 3 times that of Lipase PS-30 (2:1) for hydrolysis of diester **1**; thus, Lipase PS-SD required 3 times the enzyme loading compared with Lipase PS-30 for reaction completion in the same amount of time.

Immobilized Lipase B from C. antarctica, Novozym 435. Though the Lipase PS-SD enzyme-catalyzed hydrolysis provided about 100 kg of monoester **2** for drug development studies, we still desired a better enzyme that could catalyze the hydrolysis at a much lower enzyme loading. Furthermore, some emulsion was encountered during the workup of both the Lipase PS-30 and Lipase PS-SD-catalyzed hydrolysis procedures, and either Celite pad filtration or centrifugation had to be employed to break up the emulsion. We thought that an immobilized enzyme such as Novozym 435, where the protein is immobilized on an insoluble carrier, might solve the emulsion issue. Novozym 435, a product of Novozymes, is lipase B from *C. antarctica* immobilized on a macroporous resin. During the initial enzyme screening, Novozym 435 had been identified as a promising enzyme on the basis of its activity, but it was not selected because of the increased level of diacid **3** formation compared with Lipase PS-30. It was later found (see below) that the formation of diacid **3** by Novozym 435 could be minimized by appropriately controlling the substrate to enzyme ratio, reaction pH, and reaction temperature.

Process Development Using Novozym 435. The Novozym 435-catalyzed hydrolysis was evaluated on a 40 mg substrate scale in 1 mL of sodium phosphate buffer (0.2 M, pH 7) at 40 °C for 24 h at various substrate to enzyme ratios. The enzyme loading had a significant effect on the reaction rate and the amount of diacid **3** formation. As expected, more diacid was formed at high enzyme loading. The 1 mL scale experiments were performed without pH adjustment during the reaction, and the pH dropped to ~5 as the hydrolysis progressed. The desired monoester **2** was probably not ionized completely at pH ~5 and was likely to remain adsorbed on the nonpolar acrylic beads of the immobilized enzyme, thus undergoing further hydrolysis to diacid **3**. This was probably the major reason for the higher levels of diacid **3** seen during enzyme screening. It was especially important to maintain the pH close to 7 to minimize further hydrolysis of monoester **2** to diacid **3** when using an immobilized enzyme. Eight commonly used cosolvents (10% v/v in buffer) were evaluated, and as with the lipase PS-SD, the addition of cosolvent did not show any benefit and was not pursued.

Further optimization of the Novozym 435-catalyzed hydrolysis of diester **1** was performed on a 1 g scale at a substrate concentration 50 g/L. Various enzyme to substrate ratios and reaction temperatures were evaluated. Diester **1** immediately adsorbed onto the immobilized enzyme beads and remained as a large sticky mass in the reaction mixture. Interestingly, the adsorption of the substrate on the immobilized enzyme beads became less pronounced as the reaction temperature was increased from 40 to 60 °C. A maximum operating temperature of 60 °C was recommended[22] for optimum productivity with Novozym 435. The optimum temperature for the hydrolysis of **1** was established to be 55 °C. Diacid formation at 60 °C was only slightly higher than that at 55 °C, and thus, a slight excursion of temperature above 55 °C should not be detrimental. The optimum pH for the reaction was 7, and the pH was kept constant during the reaction by addition of NaOH as needed. The optimum enzyme to substrate ratio was 1:50, requiring only 1 g/L Novozym 435 to hydrolyze 50 g/L diester **1**. The workup was significantly easier than the previous processes with Lipase PS-30 or Lipase PS-SD with no emulsion issues.

Scale-Up of the Process Using Novozym 435. The Novozym 435-catalyzed hydrolysis was scaled up with increasing amounts of diester **1** up to a 500 g scale. The diester **1** for the 500 g scale reaction was crude, with an HPLC (UV 210 nm) AP of 41, and contained several impurities originating from its synthesis. One impurity was identified as 2,6-di-*tert*-butyl-4-methylphenol (BHT) with an AP of 35. There were other unknown impurities, the highest being the one with an AP of 6, and several unknown impurities each with AP as high as 2. BHT and probably other impurities have stronger UV absorption and HPLC areas, causing their amounts to be overestimated. The potency of the diester **1** was high (90%), and it was used as such for the enzyme reaction without purification. The enzyme-catalyzed hydrolysis of diester **1** (500 g) was carried out with 10 g of Novozym 435 under the optimum reaction conditions at 55 °C and pH 7 with manual pH adjustment. The composition of the reaction mixture at various times is shown in Figure 1. At 31 h, the

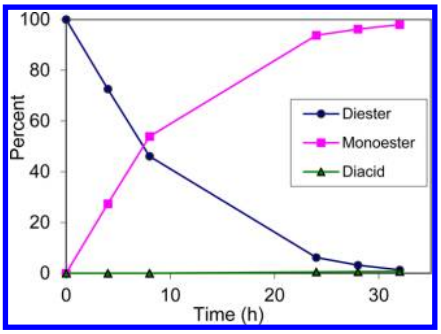

Figure 1. Enzymatic hydrolysis of diester **1** to monoester **2** by immobilized lipase B from *C. antarctica* (Novozym 435).

monoester **2**:diester **1**:diacid **3** ratio was 97.9:1.5:0.6. Extractive workup gave 370.7 g of monoester **2** (93% potency, 82% "as is" yield, 91% yield corrected for the potency of the starting diester). HPLC showed good purity, with APs of 86.1 for monoester **2** and 0.3 for diacid **3**, no detectable diester **1**, no detectable BHT, and a total AP of 13.6 for eight impurities, all coming from the starting material. The crude monoester **2** was successfully applied for the subsequent synthetic step where these impurities were purged.

Reuse of Novozym 435. In order to demonstrate the potential for recycling, Novozym 435 was collected by filtration after one reaction. The collected Novozym 435 was sticky, which may be due at least in part to the adsorption of impurities present in the diester on the immobilized resin. The

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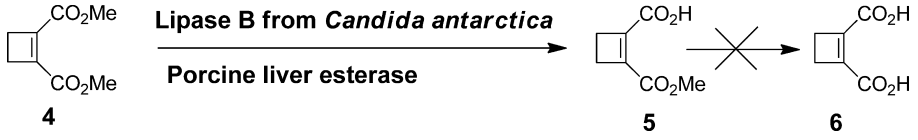
Scheme 2. Hydrolysis of Dimethyl Cyclobut-1-ene-1,2-dicarboxylate (4)



recovered Novozym 435 could not be reused as such. It was found that washing with MTBE gave a free-flowing material, probably by removing the impurities from the resin surface. After the MTBE wash, the recovered enzyme was dried in a vacuum oven at room temperature overnight to remove residual MTBE. The recovered enzyme (300 mg) was used to hydrolyze 12 g of diester **1** under the same optimum conditions. The conversion reached 93% in 54 h with a 0.9% yield of diacid **3**. The recovered enzyme showed the same selectivity as the fresh enzyme but only about 60% of the original activity. MTBE washing might have reduced the enzyme activity. Other solvents for washing can be explored to minimize loss of activity. However, reuse of enzyme requiring such a low loading (enzyme to substrate ratio 1:50) is not a significant concern in this case and was not pursued further.

Hydrolysis of Dimethyl Cyclobut-1-ene-1,2-dicarboxylate (4) to Monoester 5. Like the bicycloheptane diester **1**, conventional chemical hydrolysis of dimethyl cyclcobut-1-ene-1,2-dicarboxylate (**4**) to the monoester **5** (Scheme 2) was difficult without significant hydrolysis to the diacid **6**. Selective methylation of diacid **6** by DBU and iodomethane was reported to provide monoester **5** in 66% yield.[23] Screening of enzymes for the hydrolysis of **4** to monoester **5** (Scheme 2) identified Novozym 435 as one of the best. Interestingly, porcine liver esterase (PLE) was found to be even better than Novozym 435. Both hydrolyses afforded almost no diacid **6**. The optimum reaction conditions with both enzymes were developed and used to carry out larger-scale reactions. The hydrolysis of diester **4** at 50 °C for 4 h catalyzed by Novozym 435 with an enzyme to substrate ratio of 1:20 and a simple isolation method provided monoester **5** in 78% yield with excellent quality (monoester **5**, AP = 99.3; remaining diester **4**, AP = 0.7; diacid **6** not detected). Hydrolysis of diester **4** at 40 °C for 5.5 h catalyzed by PLE with an enzyme to substrate ratio of 1:200 and a simple isolation method provided monoester **5** in 87% yield with excellent quality (monoester **5**, AP = 99.7; remaining diester **4**, AP = 0.3; diacid **6** not detected).

In summary, monoester **2** was prepared by hydrolysis of dimethyl bicyclo[2.2.1]heptane-1,4-dicarboxylate (**1**) with a lipase from *B. cepacia* and immobilized lipase B from *C. antarctica*. The immobilized lipase B from *C. antarctica* and porcine liver esterase were also efficient for the selective hydrolysis of dimethyl cyclobut-1-ene-1,2-dicarboxylate (**4**) to the corresponding monoester **5**.

EXPERIMENTAL SECTION

Diester **1** was purchased from commercial suppliers and prepared on a large scale as described in the literature.[3−7] Diester **4** was purchased from commercial suppliers. Lipases PS-30 and PS-SD from *B. cepacia* were purchased from Amano. Immobilized Lipase B from *C. antarctica* (Novozym 435) was purchased from Novozymes. PLE was purchased from Sigma. Other commercial enzymes were purchased from their suppliers.

HPLC Methods. The conversion and the amounts of substrates and products in the reaction mixture were determined from the relative area percentages (APs) of the HPLC peaks without any correction. HPLC area versus concentration standard curves for authentic standards were used to determine the potencies of various batches of diesters and monoesters.

Two reversed-phase HPLC methods were used to monitor hydrolysis of **1**. Both employed a Waters XTerra RP-18 column (3.5 μm, 150 mm × 4.6 mm) at ambient temperature with UV detection at 210 nm with a flow rate of 1 mL/min of solvent A (0.05% TFA in 80:20 water/methanol) and solvent B (0.05% TFA in 80:20 acetonitrile/methanol), but they used different gradients. HPLC method 1 was used for enzyme screening with a gradient from 20% to 70% solvent B over 8 min. The retention times were 3.4 min for diacid **3**, 5.2 min for monoester **2**, and 7.0 min for diester **1**. HPLC method 2 was used to analyze the reaction progress and impurity profile with a gradient from 0 to 100% solvent B over 20 min. The retention times were 8.0 min for diacid **3**, 10.1 min for monoester **2**, and 12.0 min for diester **1**.

HPLC method 3 was used to monitor the hydrolysis of **4** using a Waters XTerra RP-18 column (3.5 μm, 50 mm × 4.6 mm) at ambient temperature with UV detection at 220 nm with a flow rate of 2 mL/min with solvent A (0.05% TFA in 5:95 acetonitrile/water) and solvent B (0.05% TFA in 95:5 acetonitrile/water), employing 0% B for 2 min followed by a gradient from 0 to 100% B for 2 min. The retention times for diester **4**, monoester **5** and diacid **6** were 3.0, 1.5, and 0.6 min, respectively.

Enzyme Screening for Hydrolysis of Diester 1 or 4 to Monoester 2 or 5, respectively. Sodium phosphate buffer (0.1 M, pH 7, 1 mL) was added to each well of multiwell plates, with each well containing about 10 mg of enzyme. Each plate was shaken in a Thermomixer R shaker at 600 rpm and 25 °C for 30 min. A solution containing 4 mg of diester **1** in 20 μL of DMSO was added to each well. The enzymatic hydrolysis was conducted by shaking the plate in the same shaker for 24 h at 30 °C. To each well were added 50 μL of 1 M HCl and 1 mL of methanol. After 5 min of mixing, the mixture was filtered through a 0.2 μm filter and analyzed by HPLC.

A solution of 10 mg of diester (**1** or **4**) in 50 μL of DMSO was used for some enzymes. For immobilized enzymes or enzyme solutions that were not in any multiwell plate format, the screening experiments were carried out in 4 mL vials using the same buffer. The vial was placed in the multiwell plate, and the plate was shaken in a Thermomixer R shaker at 30 °C for 24 h before analysis in the same way as before.

Optimization of Lipase PS-30-Catalyzed Hydrolysis of 1 on a Small Scale: Effects of Cosolvent, Temperature, and pH. The experiments were carried out in 4 mL vials. To each vial were added diester **1** (20 mg), Lipase PS-30 (2, 10, or 20 mg), 1 mL of sodium phosphate buffer (0.1 M, pH 6, 7, or 8), and an organic solvent or water (100 μL). The vials were placed on multiwell plates, and the plates were shaken in a Thermomixer shaker at 600 rpm at different temperatures (30, 35, or 40 °C). After 24 or 65 h, 50 μL of 1 M HCl and 3 mL of

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methanol were added to the whole reaction mixture, followed by filtration through a 0.2 μm filter and analysis by HPLC.

Lipase PS-30-Catalyzed Hydrolysis of Diester 1. To a 500 mL jacketed reactor equipped with a pH meter and an overhead stirrer were added 10 g of pure diester **1**, 5 g of Lipase PS-30, and 200 mL of sodium phosphate buffer (0.1 M, pH 7.0). The temperature was maintained at 40 ± 1 °C using a water circulator. The mixture was stirred at 300 rpm. The pH dropped slowly and was adjusted back to 7.0 periodically by addition of 5 M NaOH (every 2 h during the first 8 h, no adjustment between 8 and 24 h, and then every hour from 24 to 31 h. Samples (200 μL) of the reaction mixture were taken out with a wide-bore pipet tip at various times, mixed with 20 μL of 1 M HCl and 3 mL of MeOH, filtered, and analyzed by HPLC. At 31 h, about 2.8% of diester **1** remained. The mixture was cooled to about 23 °C, adjusted to pH 8.0 with 5 M NaOH, and extracted with 300 mL of MTBE. The MTBE layer containing the unreacted diester **1** was discarded. The aqueous phase was immediately adjusted to pH 2 using 5 M H_2SO_4 and then extracted twice with MTBE (300 mL for the first time and 200 mL for the second). The MTBE phase contained some emulsion and was filtered through a thin pad of Celite to help with phase separation. Removal of solvent from the combined MTBE phases gave 8.63 g of monoester **2** as a white solid (92.4% yield, AP = 99.7) along with an unknown impurity (AP = 0.3), and neither diester **1** nor diacid **3** was detected.

Lipase PS-SD-Catalyzed Hydrolysis of Diester 1. To a 1 L jacketed reactor equipped with a pH meter and an overhead stirrer were added 20 g of diester **1** (crude, AP = 60, 87% potency), 30 g of Lipase PS-SD, and 400 mL of sodium phosphate buffer (0.2 M, pH 7.0). The temperature was maintained at 48 ± 2 °C. The mixture was stirred at 300 rpm. The pH dropped slowly and was adjusted back to 7.0 periodically by addition of 5 M NaOH. At 25 h, the conversion reached 97.4% with 2.6% of the diester **1** remaining and a negligible amount of diacid **3**. The reaction mixture was cooled to about 23 °C, adjusted to pH 8.0 with 5 M NaOH, and then extracted with 300 mL of MTBE. Removal of solvent from this MTBE extract gave 2.2 g (11% of the starting amount) of material containing unreacted diester **1** (no monoester **2** or acid **3**) and other impurities present in the starting crude diester. The aqueous phase was immediately acidified and extracted with MTBE (2 × 400 mL). The MTBE phase contained some emulsion and was filtered through a thin pad of Celite to help with phase separation. The combined MTBE phases were washed with 100 mL of water, concentrated to dryness, and further dried in a vacuum oven at 30 °C overnight to give 15.26 g of monoester **2** (AP = 95, 82% "as is" yield), and neither the diester nor the diacid was detected. A total HPLC AP of 5 was due to nine unknown impurities coming from the starting material. The corrected yield for this batch calculated from the "as is" yield divided by 0.87 (the potency of the crude starting diester) was 94%.

Optimization of Novozym 435-Catalyzed Hydrolysis of Diester 1. Initial studies to explore the best reaction conditions using Novozym 435 were done on a 1 mL scale in 4 mL vials, which were followed by a 1 g scale. The best conditions developed are as follows. To the reactor were added 20 mg of Novozym 435, 1 g of diester **1**, and 20 mL of sodium phosphate buffer (0.2 M, pH 7.0). The mixture was stirred with a magnetic stir bar. The temperature was maintained at 55 ± 2 °C. The pH dropped slowly and was adjusted back to 7.0 using aqueous 5 M NaOH each hour during the first 8 h. After 8 h, the pH dropped slowly and was not adjusted. Samples were taken periodically and analyzed in the same way as described above. At 23 h, HPLC analysis showed that the reaction mixture contained 0.4% diester **1**, 98.9% monoester **2**, and 0.7% diacid **3**.

Novozym 435-Catalyzed Hydrolysis of Diester 1. The diester **1** had a potency of 90% and an HPLC (UV 210 nm) AP of 41. There was a major impurity of BHT with AP = 35, another unknown impurity with AP = 6, and several other unknown impurities each with an AP as high as 2. To a 20 L jacketed reactor equipped with an overhead stirrer, a pH meter, and a water circulator were added 10 g of Novozym 435, 500 g of crude diester **1**, and 10 L of sodium phosphate buffer (0.2 M, pH 7.0). The mixture was stirred at 150 rpm while being gradually heated to and maintained at 55 ± 2 °C. At the beginning, Novozym 435 resins were floating on the top, and they gradually sank into the buffer mixture in about 1 h. The diester **1** appeared as a slurry suspension of particles at the start, gradually became a sticky gel, and finally melted into fine droplets. The pH of the reaction mixture dropped slowly with the progression of hydrolysis and was manually adjusted back to 7.0 with 5 M NaOH every 30 min during the first 4 h, every hour during the following 4 h, and every 2 h until 12 h. After 12 h, the pH dropped slowly and was not adjusted until 23 h. The pH was again adjusted back to 7.0 with 5 M NaOH at 23 and 28 h. Samples were taken periodically and analyzed as described before. At 31 h, HPLC showed that the reaction mixture contained 97.9% monoester **2**, 1.5% diester **1**, and 0.6% diacid **3**. The stirring was stopped, and the reactor was cooled to 4 °C for 16 h. This holding is not necessary if the workup can be done immediately.

The mixture was warmed to 20 °C, adjusted to pH 8.0 with 5 M NaOH, and filtered through a Buchner-style M porosity filter funnel. The immobilized enzyme residue was washed with 1 L of phosphate buffer (0.2 M, pH 8.0). The combined filtrate and washing were twice extracted with 5 L of MTBE to remove remaining unreacted diester **1** along with some impurities coming from the starting material. The aqueous phase was immediately adjusted to pH 2.5 with 5 M H_2SO_4 (467 mL) and twice extracted with 5 L of MTBE. The acidic MTBE extracts were combined, washed with 3.5 L of water, and concentrated on a rotary evaporator to give a white solid, which was further dried in a vacuum oven at 30 °C overnight to give 370.7 g of monoester **2** (93% potency, 82% "as is" yield, 91% yield corrected for the potency of the starting diester). HPLC showed good quality of monoester **2** (AP = 86.1), a small amount of diacid **3** (AP = 0.3), no detectable diester **1**, no detectable BHT, and eight impurities all coming from the starting material (total AP of 13.6). The crude monoester **2** was successfully applied in the next synthetic step, where these impurities were purged.

Novozym 435-Catalyzed Hydrolysis of Diester 4. To a 2 L jacketed reactor equipped with an overhead stirrer, an addition funnel, and a pH meter were added 3.0 g of Novozym 435 and 1200 mL of sodium phosphate buffer (0.2 M, pH 7.0). The reaction temperature was maintained at 50 ± 2 °C. After the inside temperature rose to 50 °C, 60.0 g of diester **4** (353 mmol) was added. The mixture was stirred, and the pH was maintained at 6.8−7.1 by addition of 1 M NaOH as necessary. Samples of the reaction mixture (100 μL) were taken out periodically using a wide-bore pipet tip, mixed with 20 μL of 1 M HCl and 10 mL of MeOH, filtered through a 0.2 μm filter, and analyzed by HPLC. The amounts of diester **4**, monoester

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5, and diacid 6 after 4 h were 2.7%, 88%, and 9.3%, respectively. After 4 h the reaction was stopped, and the immobilized enzyme was filtered out. The filtrate was extracted with EtOAc, and the EtOAc layer containing the unreacted diester 4 was discarded. The aqueous layer was acidified to pH 3.0 with 6 M HCl (45 mL) and extracted with 900 mL of EtOAc. The pH of the aqueous phase was pH 3.8 after the first extraction. To the aqueous phase was added 150 g of NaCl, and the pH was adjusted to 3 with 6 M HCl (15 mL) before a second extraction with 600 mL of EtOAc. Again, the aqueous phase was adjusted to pH 3.0 with 6 M HCl (10 mL) and then was extracted a third time with 600 mL of EtOAc. The aqueous phase was analyzed after each extraction to ensure complete extraction of the monoester into the organic phase. Each successive extraction showed reduced levels of monoester in the aqueous phase. After the third extraction, the aqueous phase contained only diacid with a very small amount of monoester and no diester. The aqueous phase was discarded after the third extraction.

The combined EtOAc extracts were filtered through filter paper. Removal of solvent on a rotary evaporator at 40 °C gave a liquid (62 g), to which 500 mL of heptane was added under stirring. The product monoester first oiled out and then solidified. The mixture was stirred overnight at room temperature. The mixture was filtered through filter paper. The solid was collected, set in a hood for 1 h, and dried in a vacuum oven at 30 °C overnight to give 41.4 g of the first crop of monoester 5 (75.2% yield, AP = 99.3) a small amount of diester (AP = 0.7); the diacid was not detected. The filtrate was stirred in an ice bath for 1 h and some white solid formed. The solid was collected by filtration through filter paper, set in a hood for 1 h, and dried in a vacuum oven at 30 °C overnight to give the second crop of monoester 5 (1.5 g, 2.7% yield, AP = 99.6) with a small amount of diester (AP = 0.3); the diacid was not detected.

PLE-Catalyzed Hydrolysis of Diester 4. To a 5 L jacketed reactor setup with an overhead stirrer, a pH meter, and a water circulator was added 750 mg of PLE (41 units/mg) followed by 1.5 L of sodium phosphate buffer (0.2 M, pH 7.0). The temperature was maintained at 40 °C using a circulating water bath. The mixture was stirred for 20 min to dissolve the enzyme. To the enzyme solution was added 150 g of solid diester 4 (0.88 mol). The pH of the reaction mixture was maintained at 7.0 by periodic addition of 2 M NaOH. Samples (50 μL) of reaction mixture were taken out, mixed with 20 μL of 1 M HCl and 10 mL of MeOH, filtered through a 0.2 μm filter, and analyzed by HPLC to follow the conversion. At 5.5 h, the amounts of diester 4, monoester 5, and diacid 6 were 0.5%, 94.4%, and 5.1%, respectively. The pH of the reaction mixture was adjusted to 7.5 with 2 M NaOH (35 mL), and then the mixture was extracted with 1 L of EtOAc. Some emulsion formed in the organic layer, which was filtered through a pad of Celite to help with phase separation. The EtOAc layer contained the unreacted diester 4 and was discarded. The aqueous layer was acidified to pH 3.0 with 6 M HCl (160 mL) and extracted with 1.5 L of EtOAc. The aqueous layer showed a pH of 3.8 and was readjusted to pH 3.0 with 6 M HCl and then extracted second time with 1.5 L of EtOAc. The aqueous layer showed a pH of 4.0 and was again adjusted to pH 3.0 with 6 M HCl and then extracted a third time with 1 L of EtOAc. The aqueous layer after three extractions contained mostly the diacid 6. HPLC showed about 3% loss of monoester 5 in the aqueous layer, from which diacid 6 was recovered as described below.

The combined EtOAc extracts were filtered through filter paper. Removal of solvent on a rotary evaporator at 30 °C gave a solid, which was transferred into a dish and dried in a vacuum oven at 30 °C overnight to give 120.2 g of monoester 5 as white solid (AP = 99.7, 87.3% yield).

The aqueous phase was further acidified to pH 0.1 with concentrated HCl, and diacid 6 was extracted with MTBE. Crystallization from MTBE solution gave 2.5 g of diacid 6 as a white solid (AP = 97).

■ ASSOCIATED CONTENT

Supporting Information

NMR spectral data of compounds 1−6 and Tables 2−4 containing detailed data on the hydrolysis of diester 1 with Lipase PS-30, Lipase PS-SD, and Novozym 435 showing the effects of temperature, pH, substrate and enzyme input, and organic solvents. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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