Comparison of a batch and flow approach for the lipase catalysed resolution of a cyclopropyl ester; a key building block for the synthesis of ticagrelor.

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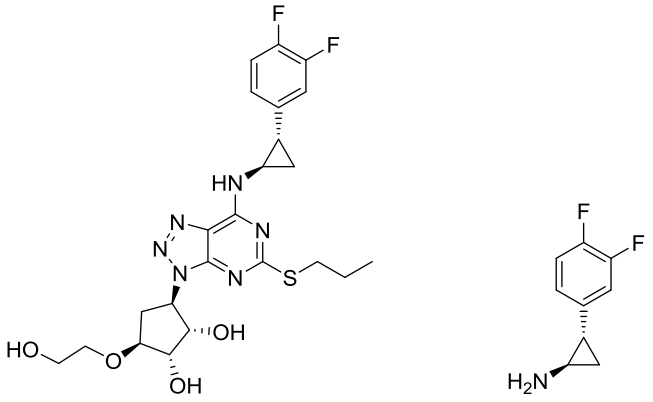
ABSTRACT: In this study a batch reactor process is compared to a flow chemistry approach for lipase catalysed resolution of the cyclopropyl ester (±)-**3**. (1*R*, 2*R*)-**3** is a precursor of the amine (1*R*, 2*S*)-**2** which is a key building block of the API ticagrelor. For both flow and batch operation, the biocatalyst could be recycled several times, whereas in the case of the flow process the reaction time was significantly reduced.

Introduction

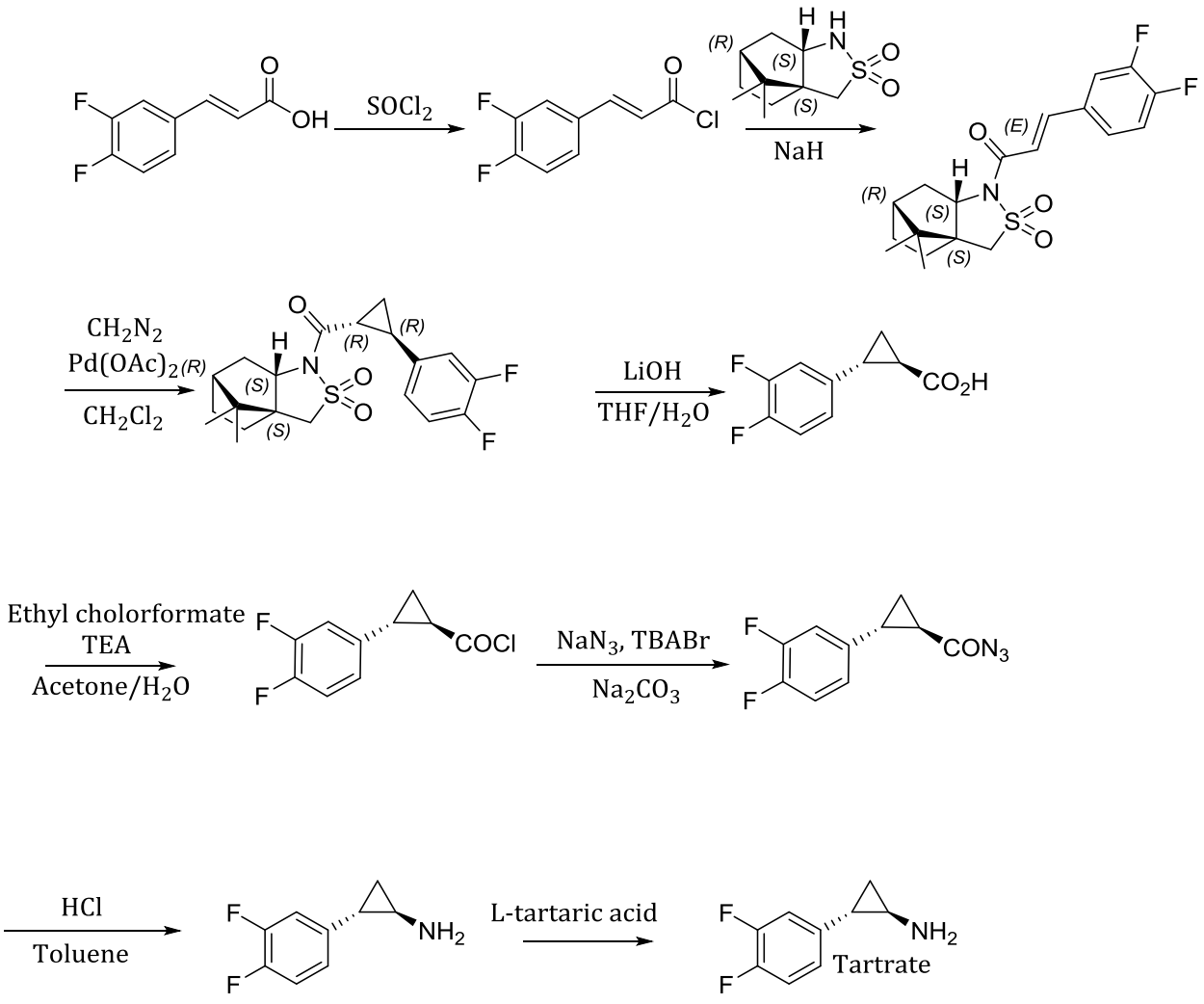
The purine analogue ticagrelor **1** is one of the world's leading medications for the treatment of acute coronary symptom and strokes. Ticagrelor acts by antagonising adenosine diphosphate (ADP) activation of G-protein coupled receptors on the surface of thrombocytes which are involved in the aggregation of platelets; this approach is commonly employed to reduce the risk of blood clotting in patients with cardiovascular diseases[1]. Ticagrelor **1** significantly reduces the frequency of death, compared to Clopidogrel the previously suggested treatment for acute coronary infarcts[2].



1 **2**

Figure 1: Ticagrelor (**1**) and the target intermediate (1*R*, 2*S*)-2-(3,4-difluorophenyl)cyclopropan-1-amine (**2**).

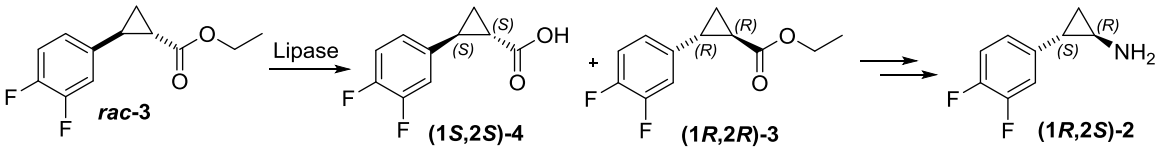
Current synthetic approaches for the preparation of the cyclopropyl amine subunit **2** of ticagrelor require multiple reaction steps and involve the use of metal catalysts and chiral auxiliaries (Scheme 1)[3–7].



Scheme 1: Synthetic route towards the target amine **2** as described in the original AstraZeneca patent[3].

In order to avoid waste generating synthetic steps, biocatalysts are increasingly applied for the generation of enantiomerically pure chiral APIs.[8] In this context, application of biocatalytic

retrosynthesis[9] identified a synthetic strategy towards the cyclopropyl subunit (1*R*, 2*S*)-**2** starting from the racemic cyclopropyl ester **3** (Scheme 2), a low value precursor easily prepared on large (kg) scale[10]. Recently we reported[11] the lipase mediated kinetic resolution of (±)- **3**, under biphasic conditions, wherein the substrate ester **3** was hydrolysed to yield the desired ester (1*R*, 2*R*)-**3** and the by-product acid (1*S*, 2*S*)-**4** (Scheme 2). Under these conditions the acid (1*S*, 2*S*)-**4** is extracted into the aqueous phase and the unreacted ester (1*R*, 2*R*)-**3** can be recovered from the organic phase and subsequently transformed into the target amine (1*R*, 2*S*)-**2** via a stereospecific Hoffman rearrangement[5,6].



Scheme 2: Enantioselective lipase-mediated hydrolysis of *rac*-**3** to yield the acid (1*S*, 2*S*)-**4** and the remaining ester (1*R*, 2*R*)-**3** which can be converted to the target amine (1*R*, 2*S*)-**2**.

As reported previously, the lipase from *Thermomyces lanuginosa* was found to efficiently catalyse the kinetic resolution of substrate ester **2** with high enantioselectivity (E =100)[11]. Although enzymes are now widely used in industrial applications[12,13], a remaining challenge for use on large scale is the production cost: therefore recyclability and stability of the biocatalyst under the reaction conditions, together with ease of work-up, need to be addressed if sustainable processes for industrial application are to be developed[14]. The use of large quantities of enzyme can create waste which needs to be disposed of, while the need to extract each batch individually

requires a considerable volume of organic solvents. Both of these issues will ultimately increase the impact of the process on the environment[15] and hence biocatalysts need to be recycled in order to encourage their application in industrial process[16].

In addition, the maintenance of the structural and catalytic activity of enzymes is essential for their recyclability. Many methods exist to increase the conversion rate of an enzyme-catalysed process, such as the immobilisation of the catalyst to enhance enzyme stability, reactions in a biphasic system to prevent product inhibition[17] and the use of nanoreactors[18]. Among them, immobilisation of enzymes is the most widely used and has been successfully applied in many industrial processes for fine chemical synthesis[19–22] and has recently been shown to be applicable even for the use of whole cell catalysts[23]. Mallin *et al*.,[24] reported an extension of the enzyme immobilisation approach by connecting the immobilised enzyme to a stirring anchor in a batch reactor. This set-up facilitates recycling experiments in consecutive batch reactors as the compartment containing the enzyme is easily removed from one batch reactor and added to the next batch. Finally, flow chemistry also has the potential to overcome these problems and increase the productivity of biotransformations[25–27]. The benefits of immobilised catalysts can be further exploited in continuous flow, in terms of the ease of separation and re-use of the catalyst[28]. One method commonly used when employing immobilised catalysts in a continuous system is a packed bed reactor[29]. In this mode the reagent solution is flowed through a reactor chamber that is filled with the immobilised catalyst. Exposure of the reagent solution to the large quantity of immobilised catalyst within the reactor, can lead to a significant reduction in reaction times. Moreover, the strong shear forces and attrition experienced in a stirred tank reactor can also shorten the lifetime of an immobilised enzyme, and so a packed bed reactor represents more

attractive alternative for long-term manufacturing. The use of immobilised lipase enzymes in continuous flow has been well documented and has been shown to increase productivity and reduce reaction times[30–32]. Herein we present several biocatalytic approaches towards the target compound ester (1*R*, 2*R*)-**3** employing different enzyme systems. The scope of batch and flow reactor systems for an environmentally benign chemo-enzymatic synthetic route towards the target amine (1*R*, 2*S*)-**2** are investigated and compared.

Results and Discussion

A) Batch reactors

In order to simulate an industrial process, we set up a reaction in a stirred tank reactor at 500mg to 1g scale and compared this set up to the lab scale (50 mg) reaction. Although the liquid preparation of the lipase was preferentially used on small scale,[11] and yielded better kinetic resolution of the target ester **3**, we chose the immobilised version of the enzyme (covalently linked to immobead; Immobead 100, 9820 Units/g) throughout the study for all batch, recycling and flow reactions, in order to simplify the work-up procedure. As can be seen from Table 1 (Entry 1), the liquid preparation afforded a very good kinetic resolution of the target ester **3** on small scale (E > 200). Comparing the enantioselectivity of the liquid and immobilised preparation of the lipase on 0.5 g scale we observed a drop in enantioselectivity (Table 1, Entries 2 and 3; E = 108 for the liquid preparation and 52 for the immobilised enzyme). Therefore, our aim was to improve the process to yield an industrially viable process wherein the immobilised enzyme could be used to obtain target ester (1*R*, 2*R*)-**3** with high yield and enantiomeric excess.

Table 1: Comparison of the lipase mediated (L = liquid preparation / B = immobilised enzyme) hydrolysis of ester **3** on mg and g scale.

Entry	% ee_s[a]	% ee_p[b]	*conv [%]* [c]	E[d]	t [h]	Units	subst.[g]	vol [mL]
1[e]	18.4	99.1	15.6	>200	48	L; 10^4	0.05	5
2[f]	77.0	95.8	44.6	108	23	L; 2 x 10^4	0.5	50
3[f]	95.8	86.2	52.6	52	23	B; 5 x 10^3	0.5	50

Conditions: small scale[e]: ester **3** (50 mg, 0.22 mmol) in phosphate buffer 0.1M, pH 7.0 (5 mL). The enzyme was added to give a 2% (w/v; 100 mg enzyme suspension, 10^4 U) solution. The reactions were stirred in a glass vial at room temperature (18°C) for 48h. gram scale[f]: substrate ester 3 (500 mg, 2.21 mmol) in glycine-NaOH buffer (pH 9.0, 0.1M, 50 mL); The enzyme was added to give a 0.4% (v/v; liquid preparation; 2 x 10^4 U) or 1% (w/v, immobilised preparation; 5x10^3 U) solution. The reactions were stirred in at room temperature (18°C) for 23h using the EasyMaxTM equipment. Reactions performed with the immobilised enzyme were stirred employing a stirring anchor, whilst reactions performed with the liquid preparation were stirred using a stirrer bar.

[a,b] determined by chiral HPLC, [c] determined as $c = \frac{ee_s}{ee_s + ee_p}$, [d] determined from ee_s and ee_p.[33]

Although stable over a longer period of time, it was observed that the immobilised enzyme was gradually ground to a fine powder in any stirred reactor, using either a magnetic flea or overhead stirring. As previously discussed, batch reactor chemistry can be further developed towards a consecutive batch system. Therefore, a 'tea bag' construction was implemented, wherein the enzyme was additionally wrapped in chemically inert first aide gauze (Figure 2) and stirred in a 1:4 heptane:glycine/NaOH buffer (0.1 M, pH 9) given that the ester is soluble in heptane and the

carboxylate **4** in the buffer. The parcel was tied close with sewing string and no leakage of the biocatalyst was observed.

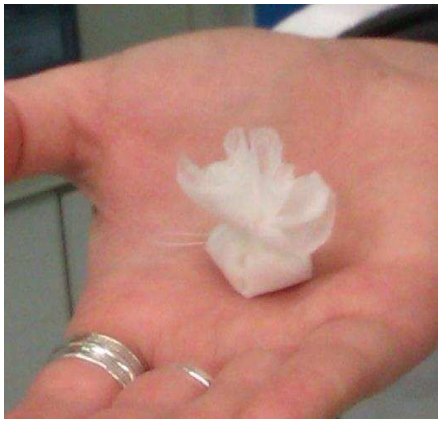


Figure 2: Immobilised TlL in first aide gauze ('tea bag')

The reaction was easily terminated by simply removing the 'tea bag' of enzyme from the bi-phasic liquid reaction medium and washing it in a mixture of heptane (to extract the remaining ester adsorbed to the beads) and fresh buffer at pH 9 (to extract the remaining acid trapped in the gauze). This set-up allowed for several cycles of hydrolysis to be performed with the same batch of immobilised enzyme without any significant loss of enzyme activity or enantioselectivity (Table 2 Entry 1 and 6). For the work-up the 'tea bag' was quickly washed in hexane and buffer prior to being added to a fresh reaction mixture.

Table 2: Batch recycling experiments using the immobilised TlL in a 'tea bag'

Entry	Cycle #	*% ee$_s$*[a]	*% ee$_p$*[b]	*conv [%]* [c]	*E*[d]
1	1	85.2	76.8	52.6	20
2	2	77.2	85.4	47.5	30
3	3	72.8	86.9	45.6	31
4	4	70.8	88.2	44.5	34
5	5	75.0	87.6	46.1	34
6	6	74.9	88.7	45.8	37

Conditions: substrate ester **3 (**500 mg, 2.21 mmol) in glycine-NaOH buffer (pH 9.0, 0.1M, 80 mL) and heptane as co-solvent (20 mL); The enzyme was added as a tea bag containing the immobilised preparation of the lipase to give a 1% (w/v; 10^4 U) solution. The reactions were stirred in at room temperature (18°C) for 23h using the EasyMax^{TM} equipment and a stirrer bar.

[a,b] determined by chiral HPLC, [c] determined as $c = \frac{ee_s}{ee_s + ee_p}$, [d] determined from ee_s and ee_p[33].

One issue that needed to be addressed was the retention of substrate and product in the 'tea-bag'. Although a short washing step allowed for most of the substrate and product to be recovered some remained in the bag, as established by analysis of the mass balance of the reaction. In order to determine the initial loss of material, a second reaction was set-up under identical conditions but the bag was thoroughly washed, extracted and the products analysed. It was found that 20% of the product remained in the tea bag after one cycle of reaction, and that this material can be recovered by a thorough washing step at the end of the reaction.

B) Flow chemistry

The set-up chosen for the flow reactions was a packed-bed system consisting of a stainless steel column reactor, packed with immobilised enzyme (immobilised TIL) (Figure 3). The reagent streams were pumped from two separate syringe pumps into a T-piece for mixing prior to entering the packed reactor. The biphasic reaction mixture was collected into a separating funnel at the outlet of the reactor for separation and subsequent re-circulation through the reactor.

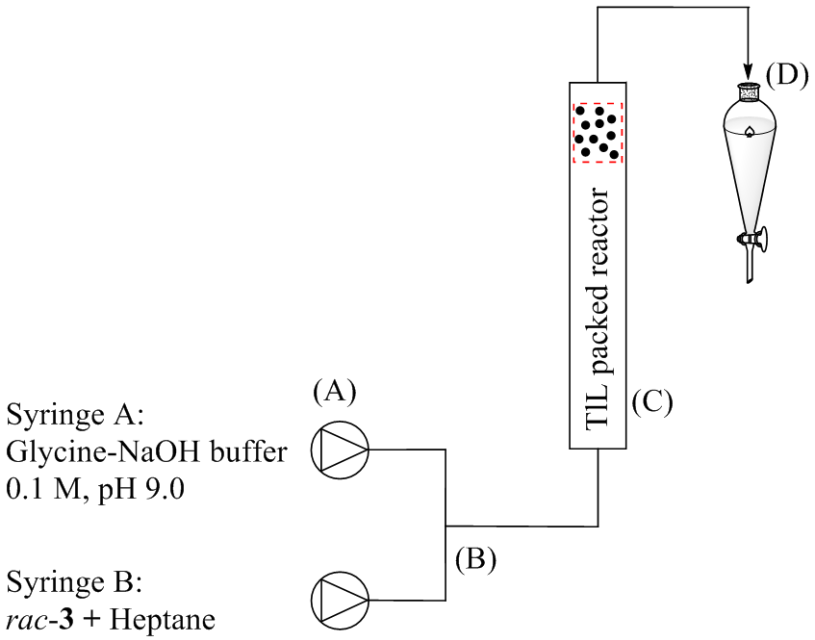


Figure 3: Schematic overview of flow chemistry set-up. (A) Syringe pump; (B) Mixing T-piece; (C) Packed column reactor (250 x 4 mm), containing immobilised TlL (shown as black spheres in the cut-out (dashed red)); (D) Product collection and separation.

The volume and mean residence time (Tres) of the reactor was determined by passing dye into the reactor and measuring the time required for it to elute from the reactor. Pure heptane and glycine-NaOH aqueous buffer (1.1 M, pH 9) were passed through the reactor at equal flow rates with a total flow rate of 0.5 mL/min. A 10 second (0.04 mL) spike of red dye was introduced to the reactor via the buffer feed. The eluent from the reactor was collected in 1 minute fractions. The dye began eluting from the reactor after 4 minutes after entering and continued to elute for a further 6 minutes (see SI for details). The maximum concentration of dye eluted between 5 and 6 minutes after the dye entered the reactor giving a median residence time of 5.5 minutes. At a total flow rate of 0.5 mL/min this gives a reactor volume of 2.75 mL.

Once the column reactor had been packed with the immobilised enzyme, initially the system was primed with the reaction solvent before introduction of the substrate. When exposed to the reaction solvent the immobilising agent was observed to swell notably causing significant back pressure and eventual blocking of the reactor. Various amounts of enzyme were tested to alleviate this increased pressure. 1 g was found to be optimal for this system and thus reactions were carried out using this loading. The reaction solvent was chosen to be a 1:1 mixture of buffer and co-solvent (heptane). The addition of 50% co-solvent allowed the beads to be constantly washed lest the adsorption of the ester to the beads cause any substrate inhibition[11]. 10 mL of each the organic and aqueous phases were pumped simultaneously through the reactor for 40 mins, equating to 7.3 reaction volumes. This arrangement allows establishment of steady-state. Both the organic and the aqueous phase were analysed by HPLC at the end of each run. The system proved to be viable, yielding the target ester (1*R*,2*R*)-**3** with slightly higher selectivity *E* =

52 in batch and 58 in flow reactor. With Tres of 5.5 min, the conversion was 17% which compares very favourably with the 24h batch reaction.

Table 3: Lipase mediated hydrolysis of ester **3** using the flow set-up, with recirculation

Entry	Run n°	*% ee*$_s$[a]	*% ee*$_p$[b]	*conv [%]* [c]	*E*[d]
1	1	17.5	96	17	58
2	2	30.5	95.5	24	58

Conditions: Flow set-up (cf. Figure 3) Syringe (A) glycine-NaOH buffer (10 mL, 0.1 M) pH 9.0; Syringe (B) *rac*-**3** (500 mg, 2.21 mmol) in heptane (10 mL); column packed with 1g immobilised lipase; 10^4 Units; 0.5 mL/min total flow rate; residence time for a single pass through the reactor = 5.5 min; total run time = 40 min i.e. 7.3 residence times. [a,b] determined by chiral HPLC, [c] determined as c = $\frac{ee_s}{ee_s + ee_p}$, [d] determined from *ee*$_s$ and *ee*$_p$[33].

To simulate a longer Tres, the eluent was recycled a further 7.3 times, (Table 3, Entry 2). For this the organic and aqueous phases were separated and re-loaded into the syringes for the second run. Despite a drop in pH to 6.5 noted after the first run, the aqueous phase was used directly. An increase of only 7% conversion was observed, perhaps due to the combined effects of by-product acid **4** enzyme inhibition, and the pH on enzyme activity; nevertheless the substrate e.e. reached a higher value than that obtained in previous trials[11]. The space time yield of the flow reactor equals 28.2 mmol L^{-1} h^{-1}, and is therefore 64 times more efficient than the batch reactor which yielded the desired product with 0.4 mmol L^{-1} h^{-1}. Using this set-up the maximum 50% conversion would be attained theoretically with Tres of 16.5 min., though

unfortunately this could not be tested as insufficient immobilised enzyme was available. To achieve the maximum 50% conversion these findings suggest three columns in series with continuous separation of **4** (contained in the aqueous phase) and addition of fresh buffer would be better than a single stage reactor.

Conclusions

The feasibility of a biocatalytic route to a key intermediate for ticagrelor, using either (i) a consecutive batch reactor or (ii) a continuous flow chemistry set-up, were demonstrated and compared. In both cases the biocatalyst was recycled multiple times without any loss of performance. A recycling system was developed from the batch reactor where the same portion of biocatalyst was then transferred between batches, eliminating the need for lengthy work up processes and enzyme recovery. The continuous flow approach gave both increased enantioselectivity and a significantly shorter reaction time, leading to increased productivity. Further development ought to lead to an improved process to this intermediate.

Material and Methods

General:

Reagents were purchased from Aldrich or Alfa Aesar and were employed without further purification. The solvents were provided by Merck and Aldrich. The solvents of HPLC grade (hexane and 2-propanol) were provided by Aldrich and Romil. Lipase from *Thermomyces lanuginose* (TlL) was bought from Aldrich: liquid preparation >100000 Units/g (exact data not available from CoA), immobilised on Immobead 100 9820 Units/g (from CoA of specific lot); 1

Unit corresponds to the amount of enzyme that liberates 1 μmol of butyric acid per minute at pH = 7.5 at 40 °C (substrate tributyrin).

High Performance Liquid Chromatography (HPLC): The enantiomeric excesses of the ester 3 and acid 4 were determined by HPLC analysis using a Chiralcel OD-H column and an Agilent 1100 system with a UV detector. The mobile phase consisted of hexane and 2-propanol in a 95:5 ratio and the addition of 0.1% trifluoroacetic acid at 20°C and was pumped through the column at a flowrate of 1 ml/min. The observed retention time for ester **3** was 5.2 min (1*R*,2*R*) and 5.6 (1*S*,2*S*) min while acid **4** eluded with a retention time of 6.9 min (1*R*,2*R*) and 8.2 min (1*S*,2*S*), respectively. Peaks were assigned to the respective enantiomers by comparison to a sample with known absolute configuration and enantioselectivities calculated from the peak areas in the chromatograms[33].

Lipase- mediated ester hydrolysis of ester **3**:

Small scale reactions were performed in phosphate buffer 0.1M, pH 7.0 (5 mL) in a 10 mL flask. The substrate ester rac-9 was added as a 1% (w/v; 50 mg) solution to the solvent. The enzyme was added to give a 2% (w/v; 100 mg enzyme suspension) solution. The reactions were stirred at room temperature (18°C) for 48h.

Gram-scale reactions were performed in glycine-NaOH buffer (pH 9.0, 0.1M) using the EasyMaxTM equipment (using overhead stirring for the beads and a stirrer bar for the liquid preparation). Substrate ester **3** and enzyme (liquid preparation or beads) were added to the buffer as indicated for each experiment and the reaction mixture was stirred at room temperature (18°C) for 23h.

Reactions using the *tea-bag system* were set up as indicated above with the alteration that 1g (10^4 Units) of immobilised enzyme was wrapped in inert first-aid gaze and the parcel added to the EasyMax™ batch reactor set up with a stirring anchor in glycine-NaOH buffer (80 mL, pH 9.0, 0.1M) and Heptane (20 mL). Substrate ester **3** (0.5 g) was added to the reaction medium and the mixture was stirred at room temperature (18°C) for 23h.

The flow chemistry set-up, as illustrated in scheme 1, was built using an empty HPLC column (250 mm x 4 mm) which was filled with the immobilised enzyme (1g, 10^4 Units). The mobile phase was pumped through the system using two Harvard 11 syringe pumps, set to 0.25 mL/min which gave a total run/residence time of 40 min. Syringe A: Glycine-NaOH buffer (10 mL, 0.1 M) pH 9.0; Syringe B: heptane (10 mL) and substrate ester *rac*-**3** (500 mg)). PTFE and Marprene tubing was used to connect the pumps to the column. The two flows were mixed via a T-piece prior to entering the column. The mixing was monitored by adding a red food dye to the buffer in syringe A.

ASSOCIATED CONTENT

Supporting Information. For setup of flow reactor and analysis of reactants cf. supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

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