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Kinetic resolution of a precursor for *myo*-inositol phosphates under continuous flow conditions

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ABSTRACT

In this work, we have investigated the biocatalytic continuous flow process with a packed-bed reactor for the kinetic resolution of (±)-1,3,6-tri-*O*-benzyl-*myo*-inositol ((±)-**1**) by alcoholysis using Novozym 435. Excellent conversions and stereselectivities were attained in short reaction time. We found that this enzymatic transformation under continuous flow in TBME with vinyl acetate (10:1 ratio with (±)-**1**) at 50 °C, with a 3 min-residence time secured the best results (50% conversion and ee_p > 99%). The feasibility of a continuous operation of the Novozym 435-containing-packed-bed reactor over a longer period of time was demonstrated via a 9-cycle experiment wherein the lipase remained stable.

1. Introduction

Myo-inositol is the scaffold of many important molecules with fundamental roles in the physiology of living organisms. For example, the mono- and polyphosphates of *myo*-inositol, including inositol phospholipids, act as secondary messenger molecules, involved in a number of biological processes, including insulin signal transduction [1], intracellular calcium control [2], reducing blood cholesterol [3], among others. The understanding of the biological roles of such *myo*-inositol derivatives in living organisms has greatly benefited from Chemical Synthesis which has provided substantial amounts of these substances and analogues as well. The continuing evolution in the molecular diversity of inositols offers new possibilities for the development of synthetic technology.

Mostly, the syntheses of bioactive chiral *myo*-inositol derivatives employ *myo*-inositol itself, due to its availability, what implies the formation of racemic intermediates. The required resolution usually relies on the use of chiral auxiliaries yielding diastereoisomeric mixtures. In some cases, two molar equivalents of these agents are incorporated. These processes are not catalytic and the separation of the diastereoisomers may be, in some cases, a difficult process. In addition, the use of chiral auxiliaries imposes an additional step to remove this chiral resolving agent, what makes the process cost ineffective. On the other hand, enzyme-catalyzed kinetic resolutions are well known for their practicality, high efficiency and selectivity. Additionally, the separation of the formed derivative from the unreacted substrate is more easily accomplished, naturally [4–7].

Among the many enzyme classes used as biocatalysts for synthetic organic transformations, lipases (triacyl glycerol hydrolases EC 3.1.1.3) stand out as they can be applied in both aqueous and nonaqueous media, have wide substrate specificity, have an excellent ability to recognize chirality, and do not require labile cofactors [7]. Notwithstanding lipases efficiency and the advantages that they offer, few reports in the literature deal with their application to inositol syntheses. So far, the cases of *myo*-inositol derivatives, with distinctive structural patterns and including triether (±)-**1** (Scheme 1), subjected to resolutions by lipases amount to only six (five types if one only considers reactions on the cyclic backbone) (vide infra) [8]. This is surprising, in some sense, due to the many possibilities that the

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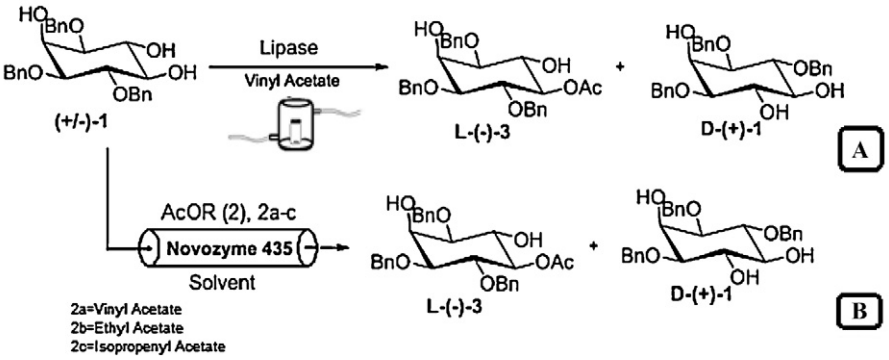

Scheme 1. Kinetic resolution of (±)-**1** by Novozym 435 (CaLB): (A) batch conditions and (B) continuous flow conditions.

myo-inositol structure offers, the importance of inositol chemistry and the broad commercial availability of lipases. A relevant feature of the lipase-catalyzed resolutions of racemic inositols is the high regioselectivities (especially in the case of acylations), which may foster synthetic flexibility and efficiency in the following functional manipulations of the unprotected polyolic moiety.

Despite the success of lipase-catalyzed reactions in batch reactors, they may suffer from long reaction times and high catalyst loadings. Such drawbacks are not found when these reactions are performed in a continuous flow system. In fact, it is reported that continuous flow systems improve the productivity of the methodology and reduce reaction times. Continuous flow systems are preferred over batch reactors due to their greater process control, high productivity and improvement of product quality/purity and yield [9,10]. Among the different types of continuous-flow reactors, packed-bed reactors (PBR) are the most popular due to their high efficiency, low cost and ease of construction, operation and maintenance [11–14].

Lipase-catalyzed kinetic resolutions in continuous-flow systems have been addressed by different reports. However, the application of this technology to the synthesis of inositols has not been previously studied.

Recently, we have developed kinetic resolutions of (±)-1,3,6-tri-*O*-benzil-*myo*-inositol, (±)-**1**, and (±)-1,2-*O*-isopropylidene-3,6-di-*O*-benzyl-*myo*-inositol using lipase B from *Candida antarctica* (CaLB) with excellent yields and enantiomeric ratios [9,15]. Nevertheless, these procedures suffer from long reaction times. Those compounds are precursors of several important inositol phosphates (e.g., 1,4,5-IP_3) and analogues. The use of derivative (±)-**1** is particularly interesting as it is available in only step from *myo*-inositol. Due to the increasing demand for more economical synthetic processes and the high productivity associated with continuous-flow systems, we engaged in applying this technology [16] to the lipase-mediated resolution of (±)-1,3,6-tri-*O*-benzyl-*myo*-inositol (±)-**1**. The results of this study are reported herein. In the previous investigation on the kinetic resolutions this inositol [9], under catalysis by CaLB in batch conditions, high selectivities were achieved when vinyl acetate, hexane, TBME and, to a lesser extent, ethyl acetate were used as solvents (vinyl acetate as acylating agent).

2. Results and discussion

As a starting point, we pursued the kinetic resolution of (±)-**1** CaLB employing ethyl acetate as solvent (vinyl acetate as acylating agent) under continuous flow (Table 1). In batch reactors,

Table 1
Kinetic resolution of (±)-**1** with vinyl acetate catalyzed by Novozym 435 in TBME at different temperatures and substrate-acyl donor ratios in the continuous flow system.

Entry	(±)-**1**/**2a**	Temp. (°C)	Residence time (min)	Conv. **3** (%)[a]	ee_p (%)[a]	E[a]
1	1:5	30	3	0.4	n.d.	–
			1.5	0.1	n.d.	–
2	1:10		3	39	99	>200
			1.5	26	96	68
3	1:15		3	40	>99	>200
			1.5	30	>99	>200
4	1:5	40	3	4	92	24
			1.5	2	n.d.	–
5	1:10		3	41	>99	>200
			1.5	30	97	98
6	1:15		3	47	>99	>200
			1.5	33	97	105
7	1:5	50	3	8	96	53
			1.5	4	92	24
8	1:10		3	50	>99	>200
			1.5	41	99	>200
9	1:15		3	50	>99	>200
			1.5	40	>99	>200
10	1:5	60	3	12	95	25
			1.5	5	92	25
11	1:10		3	50	>99	>200
			1.5	44	>99	>200
12	1:15		3	50	>99	>200
			1.5	42	>99	>200

[a] Values based on HPLC analysis. See Section 4.

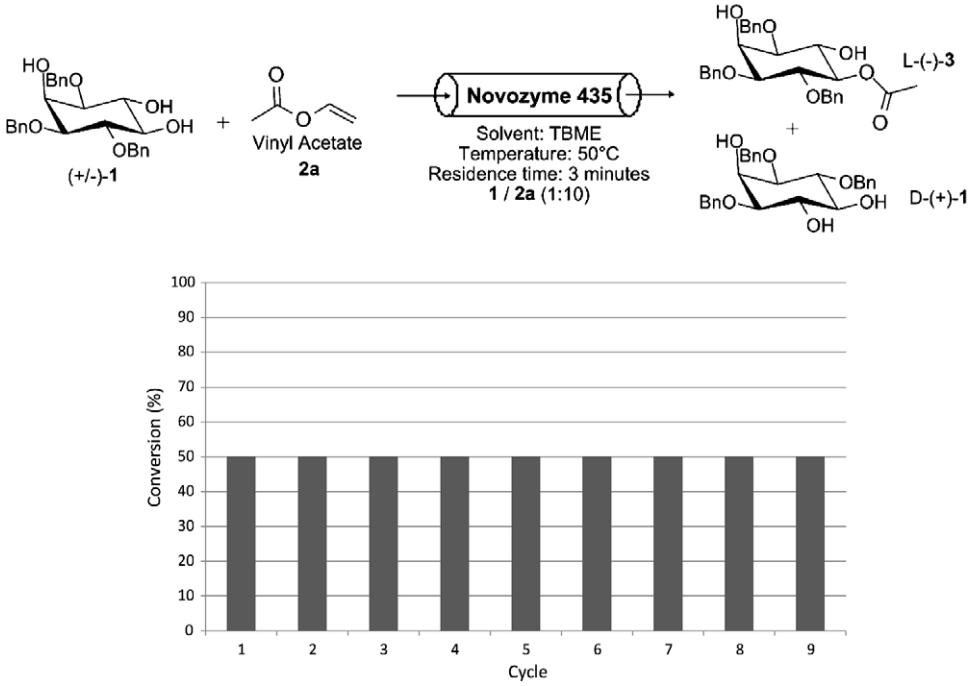

Fig. 1. Integrity of the packed-bed reactor after nine 5 min-cycles on the kinetic resolution of (±)-1 using Novozym 435 as biocatalyst, in the best experimental conditions: TBME as solvent, vinyl acetate as acyl donor (1:10 substrate/vinyl acetate ratio), at 50 °C. Residence time: 3 min.

such condition led to a stereoselective reaction showing moderate conversion (30%, $E = 39$ for the 112 h-reaction) [9]. We wondered whether, under the new condition, both conversion and stereoselectivity would be improved. Thus, a solution containing the inositol (±)-**1** and vinyl acetate in ethyl acetate was pumped through the packed-bed reactor charged with CaLB (Novozym 435). Different temperatures, residence times and proportions between (±)-**1** and vinyl acetate were tested. Unfortunately, as it had occurred in batch experiments [9], ethyl acetate did not perform well as solvent in the kinetic resolution of (±)-**1** under flow conditions. Conversions to acetate L-(−)-**3** remained below 10% even in runs applying long residence time (3 min) and large excess of vinyl acetate (1:15) at 60 °C.

We had previously shown that hexane or TBME as solvent significantly improve this transformation [9]. Due to the very low solubility of the substrate in hexane, we decided to run the continuous flow reactions in TBME (Table 1). The batch condition (with 5 mg of the substrate (±)-**1** and 0.5 U of the lipase) had led to a 47% conversion ($E > 200$) after 112 h.

The use of TBME for the kinetic resolution of *myo*-inositol (±)-**1** under continuous flow conditions had a marked effect on the efficiency of this transformation. In general, proportions of 1:10 and 1:15 (substrate/vinyl acetate) gave the best conversions/selectivity while the use of smaller excesses of vinyl acetate (1:5) led to poor conversions to the desired product and lower enantiomeric ratios (Entries 1, 4, 7 and 10, Table 1). Besides bringing about much higher productivity (vide infra) the reactions in the continuous flow system allowed the use of lower amounts of vinyl acetate compared to the batch process [9] (1:300 substrate/vinyl acetate ratio), what makes the process more economical, naturally [17].

The temperature also had an important effect on conversions, with the best results occurring at 50 and 60 °C. On the other hand, the resolutions run at 30 and 40 °C had the conversions improved by the use of larger quantities of acetylating agent (Entries 3 and 6, Table 1) or by longer residence times, with a consequent decrease on productivity. Irrespective of the chosen temperature, a residence time of 3 min gave good conversions to acetate L-(−)-**3** and higher enantiomeric excess (ee_p) and enantiomeric ratios (E) as long as a 1:10 (±)-**1**/vinyl acetate ratio, at least, was employed. It noteworthy that, when 1:10 or 1:15 (±)-**1**/vinyl acetate ratios at 50 °C under batch conditions were employed, the reaction took 24 h to achieve the same conversion and enantiomeric ratios as those achieved under continuous flow conditions as shown in Table 2.

Table 2
Kinetic resolution of (±)-**1** with vinyl acetate in TBME catalyzed by Novozym 435 at 50 °C under batch conditions. Reaction time: 24 h (productivity of 0.001 $mg_{product}/mg_{enzyme}$ h in both cases).

Entry	(±)-**1**/**2a**	Conv. **3** (%)[a]	ee_p (%)[a]	E[a]
1	1:10	49	>99	>200
2	1:15	49	>99	>200

[a] Values based on HPLC analysis. See Section 4.

The best condition obtained for the kinetic resolution of (±)-**1** with vinyl acetate under continuous flow conditions (1:10 ratio, 50 °C and 3 min of residence time) was applied to resolution with other acetylating agents in order to identify alternatives to vinyl acetate. The results obtained are shown in Table 3.

The results obtained with the use of isopropenyl acetate and acetic anhydride in TBME (Entries 1 and 2, Table 3) are good but,

Table 3
Kinetic resolution of (±)-**1** with vinyl acetate in TBME catalyzed by Novozym 435 at 50 °C in the flow system (residence time of 3 min) using different acetylating agents.

Entry	Acylating agent	Conv. **3** (%)[a]	ee_p (%)[a]	E[a]
1	Isopropenyl acetate (**2b**)	45	98	>200
2	Acetic anhydride (**2c**)	42	85	23
3	Ethyl acetate (**2d**)	3	85	12

[a] Values based on HPLC analysis. See Section 4.

when compared to our previous result using vinyl acetate (Entry 8, Table 1), show lower conversion and selectivity. In TBME, ethyl acetate as acylating agent led to very low conversion and moderate selectivity (Entry 3, Table 3).

The integrity of the biocatalyst over time was investigated by a 9-cycle assay with repeated determination of conversions (Fig. 1). Satisfyingly, the reactor displayed high consistency.

3. Conclusion

In conclusion, we have developed a very efficient continuous flow approach for the kinetic resolution of (±)-1,3,6-tri-*O*-benzyl-*myo*-inositol ((±)-**1**) by which the reactions catalyzed by Novozym 435 using TBME as solvent and vinyl acetate as acetylating agent could be performed in very short reaction times (3 min of residence time) with high conversions and enantiomeric ratios. The operation stability of the biocatalyst was demonstrated via a 9-cycle assay. The use of this protocol may lead to very practical chemical syntheses of bioactive inositols. The best condition for this flow process obtained by our screening allowed a productivity 531 times higher (3.188 $mg_{product}/mg_{enzyme}$ h) than that of the optimized condition for the corresponding batch process as we have recently established (0.006 $mg_{product}/mg_{enzyme}$ h) [21]. As a matter of fact, the productivity issue in lipase/esterase-catalyzed stereoselective syntheses of *myo*-inositols has not been addressed in the literature.

4. Experimental

4.1. Enzyme

Novozym 435 (Lipase B of *Candida antarctica* immobilized on a macroporous acrilic resin, 2720 U/g) was purchased from Novozyme. The enzyme activity was determined as the initial rate in esterification reactions between oleic acid and ethanol at a molar ratio of 1:1, temperature of 40 °C and enzyme concentration of 5 wt% in relation to the substrates [18]. One lipase activity unit (U) was defined as the amount of enzyme necessary to consume 1 μmol of oleic acid per minute at the established experimental conditions previously presented. All enzymatic activity determinations were replicated at least three times.

4.2. Continuous flow procedure

The enzymatic reactions were performed under continuous flow conditions using the Asia System from Syrris. A solution containing 4.86 mM of (±)-**1** in solvent (TBME or ethyl acetate) was used on the continuous flow system. An Omnifit column with a volume of 12.3 mL was used and fully packed with the desired enzyme. Different substrate/acylating agent ratios were used (substrate/acylating agent ratios: 1:5–15 mmol, respectively). The reaction parameters of temperature (30–60 °C) and residence time were selected on the flow reactor. Aliquots of 0.2 mL and 1.5 mL were taken for 5 min for HPLC analyses of conversion and enantiomeric excess, respectively, by HPLC. After each run was complete, we have changed the pump in order to flow pure solvent into the system in a flow rate of 5 mL/min for a period of 15 min.

4.3. HPLC analysis of conversion of (±)-1 to L-(−)-3

Conversion analyses were carried out via HPLC on a Shimadzu-C18 column (40 °C in a CTO-20A oven), eluted with an acetonitrile–H_2O (60:40) mixture (0.5 mL/min) by a Shimadzu LC-20AT pump. A Shimadzu SPD-M20A variable-wavelength UV/VIS detector was employed, with the detection set at 215 nm, and the Shimadzu LC solution software was used for chromatogram integration. The samples to be analyzed were filtered through a 0.22 μm PTFE filter. The retention times of the substrate (±)-**1** and the acetate L-(−)-**3** were 8 min and 13 min, respectively.

4.4. Determination of enantiomeric excesses (ee)

Unreacted substrate, D-(+)-**1**, and monoacetylated product, L-(−)-**3**, were separated by HPLC. In the case of substance L-(−)-**3**, it was subjected to methanolysis reaction ($MeOH/K_2CO_3$), to give triol L-(−)-**1**, prior to the HPLC analyses [19]. Chromatographic determinations of *ee* for L-(−)-**3**, ee_p (shown in the tables), (via L-(−)-**1**) were carried out on the same equipment mentioned above carrying a Chiralcel OD-H column (5 μm; 4.6 × 250 mm), eluted with a 7:3 hexane-2-propanol mixture (0.6 mL/min). The retention times of D-(+)-**1** and L-(−)-**1** enantiomorphs were 24.5 min and 28.5 min, respectively. The enantiomeric ratio (*E*) was calculated by using the equation (with ee_p and conversion as inputs; see supplementary material) of Chen et al. [20].

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2012.11.006.

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