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Screening of lipases for regioselective hydrolysis of peracetylated β-monosaccharides

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Abstract

The monodeacetylation of peracetylated-β-D-galactose (**1**) and peracetylated *N*-acetyl-β-D-glucosamine (**2**) by different lipases is here described. Lipases from different sources in an immobilized form were evaluated to find those that offer the higher activity and regioselectivity in the reactions. In the hydrolysis of **1**, the lipase from *Aspergillus niger* was the most active one, although it hydrolyzed the anomeric position. Using the lipase from *Candida rugosa*, 30% yield of the corresponding 6-OH isomer was achieved. On the other hand, in the hydrolysis of **2**, the lipase from *A. niger* was the most active and regioselective catalyst, producing more than 75% of the 6-OH derivative product.

Keywords: Lipase; Regioselective; Deacetylation; Monosaccharides

1. Introduction

Carbohydrates are inexpensive polyhydroxy-molecules with very important biological and physical functions [1,2]. Amongst them, the β-glycopyranoses maybe could be considered of high interest. For example, a high specificity for β-galactosides is shown by galectins—these are at present very important lectins due to their implication in cancer [3].

Pure regioisomers of *O*-acetyl-glycopyranoses presenting only one free hydroxyl group may be employed as key intermediates in the preparation of different glycoderivatives, such as oligosaccharides, glycolipids, glycopeptides, natural products analogues (e.g. glycosylated fraction substitution in vancomycin) [4–8] (Scheme 1).

Thus, per-*O*-acetyl-glycopyranoses could be used as raw material to obtain these building blocks. However, the use of chemical procedures implicates many protection/deprotection steps [9], because of the low regioselectivity to remove only one acetyl group among different esters with a similar reactivity.

Consequently, the use of enzymatic catalysts, especially lipases – because of their high versatility recognizing a broad range of substrates with high regio- and enantioselectivity [10–13] – could be an attractive alternative. Previous studies have reported the enzyme-catalyzed hydrolysis of different peracetylated α-glycopyranoses [14]. However, the β-peracetylated pyranoses have been rarely hydrolyzed by enzymes, very likely because of the low activity found against these compounds, as in the case of the peracetylated β-D-glucose [15].

To perform the enzymatic monodeacylation of fully acylated pyranoses, together with an acceptable activity, it is necessary to find biocatalysts exhibiting two different properties: (1) *a high specificity*—the enzyme must recognize the peracetylated monosaccharide as substrate much better than the monodeacetylated product; (2) *a high regioselectivity*—the enzyme must produce only one of the different possible regioisomers.

Here, we present a first report of the evaluation of the activity, specificity and regioselectivity of different lipases [lipases from *Pseudomonas fluorescens* (PFL), *Candida rugosa* (CRL), *Thermomyces lanuginosa* (TLL), *Rhizomucor miehei* (RML), *Candida antarctica* fraction B (CAL-B) and fraction A (CAL-A), *Aspergillus niger* (ANL) and *Aspergillus oryzae* (AOL)] [16–23] in the hydrolysis of peracetylated β-D-galactose (**1**) and peracetylated *N*-acetyl-β-D-glucosamine (**2**).

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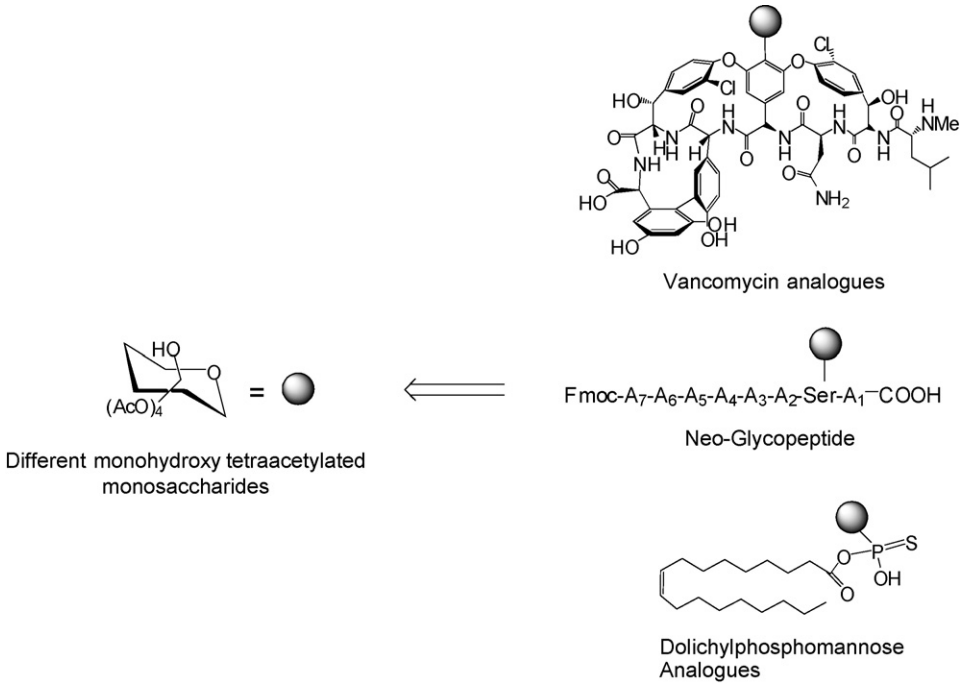


Scheme 1. Monohydroxy tetracetylated monosaccharides such as intermediates in the synthesis of different glycoderivatives.

2. Experimental

2.1. General

Lipase from *A. niger* (ANL) and *A. oryzae* (AOL) were purchased from Fluka (Neu Ulm, Germany). The lipases from *R. miehei* (Novozym 388) (RML), *T. lanuginosa* (TLL), *C. antarctica* B (Novozym 525L) (CAL-B) and *C. antarctica* A (CAL-A) were purchased by Novozymes. Lipase from *P. fluorescens* (PFL) was from AMANO. Octyl-agarose (4BCL) and cyanogen bromide (CNBr-activated sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). *C. rugosa* lipase (CRL), Triton X-100, *p*-nitrophenyl propionate (pNPP), peracetylated β-D-galactose (**1**) and peracetylated *N*-acetyl-β-D-glucosamine (**2**) were from Sigma Chem. Co. The protein concentration was determined by Bradford method [24]. HPLC analyses were performed using an HPLC spectra P100 (Thermo Separation products). The column was a Kromasil-C_8 (250 mm × 4.6 mm and 5 μm) from Analisis Vinicos (Tomelloso, Spain). Analyses were run at 25 °C using an L-7300 column oven and UV detector L-7400 at 215 nm. The eluent was an isocratic mixture of 30% acetonitrile in phosphate buffer (10 mM) at pH 4; flow rate 1.0 mL/min. Columns for flash chromatography were made up with Silica Gel 60 (Merck) 60–200 or 40–63 μm. ^{1}H NMR were recorded in $CDCl_3$ (δ, ppm) on a Bruker AMX 400 instrument.

2.2. Standard enzymatic activity assay determination

In order to follow the immobilization process, the activities of the soluble lipases and their immobilized preparations were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm ($\epsilon = 5.150\ M^{-1}\ cm^{-1}$) produced by the release of *p*-nitrophenol (pNP) in the hydrolysis of 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution (blank or supernatant) or suspension was added to 2.5 mL of substrate solution. Enzymatic activity was determined as μmol hydrolyzed pNPP/(min mg enzyme) (IU) under the conditions described above.

2.3. Purification of lipases

The purification of the lipases was performed using a previously described protocol, based on the interfacial activation of lipases on hydrophobic supports at low ionic strength [25]. 0.32 g of CRL commercial solid powder (60 mg protein), 0.5 g of ANL commercial solid powder (22 mg protein), 11 g of PFL commercial solid powder (60 mg protein), 5 mL TLL, 5 mL CAL-B, 5 mL CAL-A, 2.5 mL AOL commercial solution (12 mg protein/mL) [25] or 6.25 mL RML commercial solution (5 mg protein/mL) were dissolved in 95 mL of 10 mM sodium phosphate buffer at pH 7.0. In each case 5 g of octyl-agarose support were added. The supernatant and suspension activities were periodically checked by the method described above and the immobilization was finished after 5 h by filtration. In all cases, more than 90% of lipase was immobilized. Following this protocol, the SDS-PAGE analysis of the protein adsorbed to the octyl-sepharose [25] only showed a single band with a molecular weight corresponding to that of the different native lipases.

The lipases need to be released from the octyl-agarose afterwards, therefore it was added to a solution of 1% Triton (v/v) in 10 mM sodium phosphate buffer at pH 7.0 and 4 °C for 1 h

Table 1
Immobilization of lipases on CNBr-agarose

Enzyme	Protein loading (mg purified lipase/g support)	Immobilization yield (%)
PFL	12	100
CRL	12	100
TLL	12	100
RML	5	100
CAL-B	4.2	35
CAL-A	12	100
AOL	12	100
ANL	2.4	48

obtaining a purified lipase solution with a final concentration of 1.2 mg lipase/mL. Then the enzymatic solution was used for immobilization.

2.4. Immobilization of lipases on CNBr-activated support

Commercial agarose support activated with CNBr was suspended in an acidic aqueous solution (pH 2) during 1 h. After that the support was washed with water and dried by filtration under vacuum (eliminating only interparticle water).

Ten millilitres (for TLL, CRL, AOL, PFL, CAL-A and CAL-B), 4 mL (for ANL) and 4.2 mL (for RML) of the purified lipase solution (1.2 mg/mL) were added to 8 mL of 10 mM sodium phosphate buffer solution at pH 7. Then, 1 g of the wet CNBr-agarose support was added. The mixture was then shaken at 25 °C and 250 rpm for 18 h. After that, the solution was removed by filtration and the supported lipase was washed several times with distilled water. The percentage of immobilization grade and the amount of immobilized lipase in each case is shown in Table 1.

2.5. Enzymatic hydrolysis of peracetylated monosaccharides

Standard assay was performed as following: **1** and **2** (0.02 mmol, 8 mg) was added to 10 mL solution of phosphate buffer 50 mM with 10% acetonitrile at pH 5, 25 °C and the reaction was initialized by adding 0.8 g (**1**) and 1 g (**2**) of biocatalyst. The reaction was performed at pH 5 in order to avoid the chemical acyl-migration in the per-*O*-acetylated carbohydrates hydrolysis [15a]. The hydrolytic reaction was carried out under mechanical stirring, and the pH value was controlled by automatic titration. Hydrolysis reactions were followed by HPLC. Finally, the optimization of the reaction in each case was performed using a 8 g/L substrate and the products were isolated and identified by ^{1}H NMR.

2.5.1. 2,3,4,6-Tetra-O-acetyl-α/β-D-galactopyranose (*1a*)

1 (1 mmol, 390 mg) was hydrolyzed in 50 mL pH 5 solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-ANL preparation. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2× 10 mL), separated and dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum to afford **1a** as a white solid (373 mg, 95%). HPLC analysis: t_R = 8.3 min (β-anomer), 9.8 min (α-anomer). ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 5.52 (bd, 1H, J = 3.4 Hz, H-1), 5.48 (dd, 1H, J = 1.25 Hz, H-4), 5.41 (dd, 1H, J = 3.4 Hz, H-3), 5.19 (dd, 1H, J = 3.4 Hz, H-2), 4.72 (dt, 1H, J = 6.5Hz, H-5), 4.12–4.08 (dd, 2H, J = 11.5Hz, H-6a,b), 2.15–1.99 (s, 12H, 4× CH_3).

2.5.2. 1,2,3,4-Tetra-O-acetyl-β-D-galactopyranose (*1b*)

1 (1 mmol, 390 mg) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5g CNBr-CRL preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 40:60 hexane–ethyl acetate as eluent (117 mg, 30%). HPLC analysis: t_R = 10.6 min. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 5.73 (d, 1H, J = 8.26 Hz, H-1), 5.44 (d, 1H, J = 3.39 Hz, H-4), 5.32 (t, 1H, J = 8.37 Hz, H-3), 5.13 (dd, J = 3.42 Hz, J = 10.4 Hz, H-2), 3.91 (dt, J = 6.45 Hz, H-5), 3.8–3.51 (m, 2H, ABX system, 2× H-6), 2.14–1.97 (s, 12H, 4× CH_3).

2.5.3. 2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-α/β-D-glucopyranose (*2a*)

2 (1 mmol, 390 mg) was hydrolyzed in 50 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 8 g CNBr-PFL preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2× 10 mL), separated and dried over anhydrous Na_2SO_4, which was then removed by filtration and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 95:5 dichloromethane:methanol (97.5 mg, 25%). HPLC analysis: t_R = 6.5 min. ^{1}H NMR (500 MHz, $CDCl_3$), δ, ppm: 6.04 (d, 1H, J = 9.5 Hz, NH), 5.25 (dd, 1H, $J_{3,2}$ = 10.0, $J_{3,4}$ = 9.5 Hz, H-3), 5.15 (d, 1H, J = 3.5 Hz, H-1), 5.07 (t, 1H, J = 9.5 Hz, H-4), 4.73 (d, 1H, J = 8.5 Hz, H-1 β-anomer), 4.22 (m, 1H, H-5), 4.16 (m, 2H, H-2, H-6), 4.02 (m, 1H, H-6), 2.03 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.90 (s, 3H, N-2 CH_3).

2.5.4. 2-Acetamido-2-deoxy-1,3,4-tri-O-acetyl-β-D-glucopyranose (*2b*)

2 (2 mmol, 780 mg) was hydrolyzed in 100 mL solution of phosphate buffer 50 mM (80%) and acetonitrile (20%) using 5 g CNBr-ANL preparation at pH 5. When the substrate disappeared (checked by TLC and HPLC), the aqueous solution was filtrated and saturated with NaCl and then extracted with ethyl acetate (5× 50 mL). The collected organic layers were washed with a 5% $NaHCO_3$ solution (2× 10 mL), separated and dried over anhydrous Na_2SO_4, which was then removed by

Table 2
Specificity and regioselectivity hydrolysis of **1** catalyzed by different lipases immobilized on CNBr-agarose

Entry	Enzyme	Specific activity[a]	t (h)	c (%)	c_m [b] (%)	**1a**[c] (%)	**1b** (%)
1	PFL	0.76	96	100	96	65	14
2	CRL	0.02	90	100	66	29	37
3	TLL	0.008	168	100	77	77	
4	RML	0.0025	144	70	39	14	25
5	CAL-B	0.06	15	100	100	100	
6	CAL-A	0.042	22	100	85	85	
7	AOL	0.016	95	100	60	28	32
8	ANL	45	1	100	100	100	

[a] Specific activity was defined as μmol mg_{prot}^{-1} h^{-1}. It was calculated at 10–15% conversion.
[b] Conversion of the monohydroxy peracetylated products.
[c] Anomeric mixture α/β (60/40).

filtration and concentrated under vacuum. After that, purification by the flash chromatography column was performed with 95:5 dichloromethane:methanol (584 mg, 75%). HPLC analysis: t_R = 8.4 min. ^{1}H NMR (400 MHz, $CDCl_3$), δ, ppm: 5.80 (d, J = 3.51 Hz, H-1), 5.50 (d, J = 9 Hz, 1H-NH), 5.30 (t, J = 9.9 Hz, H-3), 5.10 (t, J = 9.6 Hz, H-4), 4.35 (dd, J = 9.8 Hz, J = 6.70 Hz, H-2), 4.28–4.20 (m, 2H, H-6), 4.19–4.10 (m, H-5), 2.21 (s, 9H, 3× CH_3), 1.96 (s, 3H, CH_3).

3. Results and discussion

3.1. Specific and regioselective enzymatic hydrolysis of peracetylated-β-D-galactose (*1*)

Several purified lipases from different sources, immobilized on agarose activated with CNBr, were studied as catalysts in the hydrolysis of **1** (Table 2, Scheme 2). We have preferred to directly study immobilized lipases instead of free enzyme to prevent possible artifacts due to the strong tendency of lipases to form bimolecular aggregates with altered properties [26].

The specific activities of the different lipases were very different against this substrate. The most active catalyst was ANL. This enzyme displayed 50 times higher specific activity compared to the second most active, PFL. Other enzymes such as CAL-B or CAL-A presented around 15 times less activity than PFL (Table 2). Other lipases were even less active, as it is the case of RML (around 18,000 times less activity that ANL).

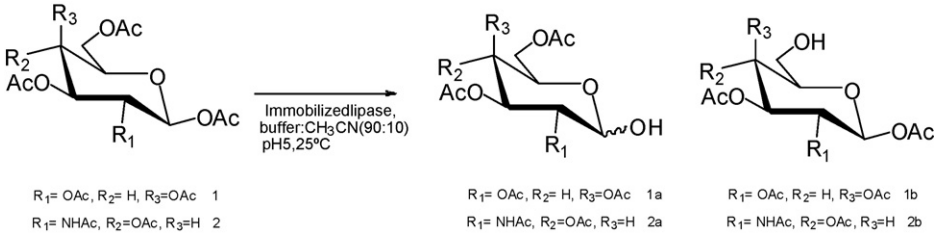
Table 2 shows the accumulation of monodeacetylated product and the regioisomers generated by the different lipases. CAL-B and ANL were quite specific and quite regioselective producing only the monohydroxy derivative with in the anomeric position (**1a**) more than 95% product conversion (Scheme 2). TLL and CAL-A were also specific towards hydrolysis of **1** although to a lesser extent than the previous enzymes, giving a conversion in **1a** of 77% and 85%, respectively (Table 2). Other immobilized lipases such as PFL, AOL and CRL produced a mixture of monodeacetylated products in anomeric (**1a**) and 6-OH (**1b**) positions. Whilst PFL showed a high specificity with 96% conversion of **1a** and **1b** in a relation 4.6:1, AOL and CRL showed a lowest product conversion (around 60%) with a relation between regioisomers of 1:1.3 (**1a**:**1b**) (Table 2). In this way, using immobilized CRL and 20 mM of **1**, it was possible to get, after purification by flash chromatography, **1b** in 30% overall yield. Moreover, **1a** was obtained directly in 95% overall yield from the enzymatic hydrolysis using immobilized ANL.

3.2. Specific and regioselective enzymatic hydrolysis of peracetylated N-acetyl-β-D-glucosamine (*2*)

Immobilized ANL was the most active catalyst towards **2**, being more than 300 times more active than CAL-A and CAL-B and more than 50,000 times compared with immobilized AOL and RML (Table 3).

In general the lipases were less active against **2** than **1** due to the presence of the acetamido group in the position 2 and also the regioselectivity was affected (Tables 2 and 3). For example, the activity of PFL decreased almost 30 times. CAL-A and CAL-B were two exceptions, the specific activity slightly increased in the hydrolysis of **2** (Tables 2 and 3).

Again, the specificity of the lipases in the production of monohydroxy products was quite different. Immobilized ANL was totally specific in the conversion of **2** in monohydroxy product



Scheme 2. Specific and regioselective hydrolysis of different peracetylated β-D-glycopyranoses.

Table 3
Specificity and regioselectivity hydrolysis of **2** catalyzed by different lipases immobilized on CNBr-agarose

Entry	Enzyme	Specific activity[a]	*t* (h)	*c* (%)	c_m [b] (%)	**2a**[c] (%)	**2b** (%)
1	PFL	0.026	168	100	84	31	48
2	CRL	0.0015	168	38	35	11	24
3	TLL	0.002	194	100	70	20	50
4	RML	0.0006	144	5	5	2	3
5	CAL-B	0.1	102	100	35	15	20
6	CAL-A	0.053	144	100	98	28	67
7	AOL	0.0006	144	6	6	2	4
8	ANL	30	1.5	100	100	20	80

[a] Specific activity was defined as μmol mg_{prot}^{-1} h^{-1}. It was calculated at 10–15% conversion.
[b] Conversion of the monohydroxy peracetylated products.

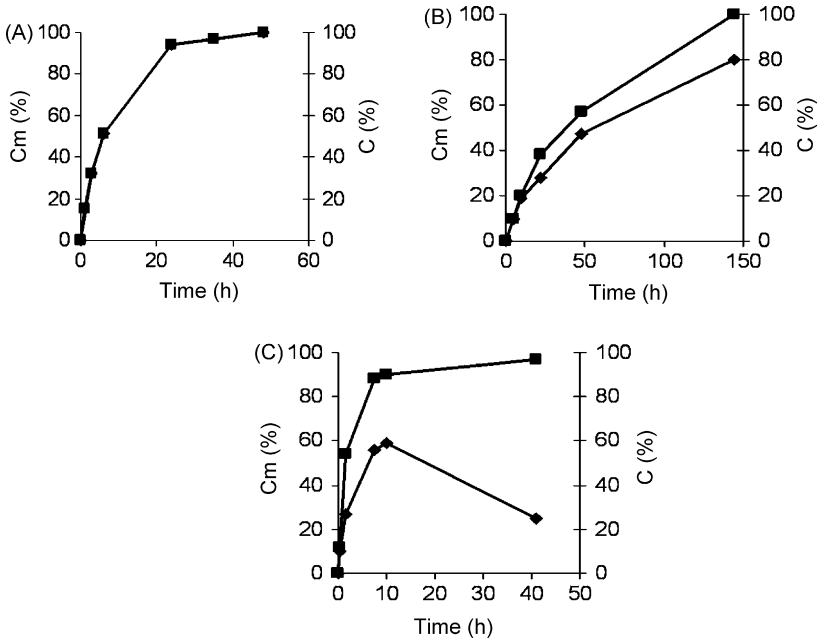


Fig. 1. Reaction Course of the hydrolysis of **2** catalyzed by different immobilized lipases: (A) CAL-A; (B) PFL; (C) CAL-B. *c*: conversion (squares), c_m: conversion of monoprotected product (rhombus).

as shown in Fig. 1A, producing 80% of **2b** and only 20% of **2a** (Scheme 2, Table 3).

When immobilized TLL or PFL were used, the monohydroxy products conversion decreased up to 70 or 80%, respectively (Fig. 1B). Using PFL, 31% of **2a** and 48% of **2b** were produced (Table 3, Scheme 2), whilst using TLL, **2a** was obtained in 20% and **2b** in 50% (Table 3). However, using immobilized CAL-B, the final monohydroxy products conversion was only 35% (Fig. 1C) in a relation of isomers of 1:1.3 (**2a**:**2b**). Therefore, when the hydrolysis was performed using immobilized ANL and 20 mM concentration of **2**, 75% overall yield of **2b** was isolated after flash chromatography purification.

4. Conclusion

This paper is the first successful report on the regioselective hydrolysis of **1** and **2** by lipases. Based on these results, we can propose a good-enough lipase for each of the processes. The immobilized ANL was the best catalyst in terms of activity towards the hydrolysis process and also highly specific and regioselective in the hydrolysis of **1**, producing the monohydroxy product **1a** in 95% yield. Other enzymes such as PFL, AOL, CRL or RML were also specific in the hydrolysis of **1** with a high regioselectivity towards production of the monohydroxy derivatives **1a** and **1b**, making it possible to obtain 30% overall yield of **1b** by using CRL. When PFL was used in the hydrolysis of **2**, the monohydroxy product **2a** was produced specifically in 25% overall yield. However, ANL, apart from the high activity displayed (100% conversion in 1 h) permitted to get **2b** in 75% overall yield. Other enzymes such as TLL, AOL or RML displayed a very low reaction rate, being quite unspecific for **2**.

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