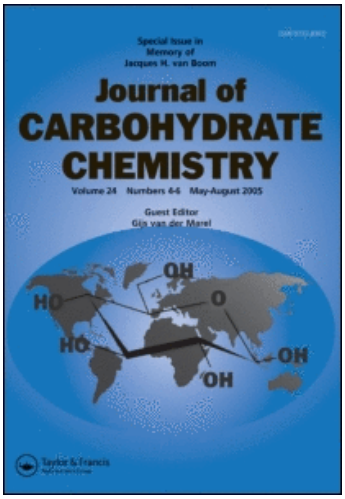

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Application of Site-Directed Lipase Mutants on Regioselective Acylation of Monosaccharides

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Application of Site-Directed Lipase Mutants on Regioselective Acylation of Monosaccharides[†]

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ABSTRACT

Mutants F344V and F345V of *Candida rugosa* lipase1 (CRL1) were tested in acylation reactions of monosaccharide derivatives **1**–**8**, in order to study the regioselectivity, and the substrate specificity of lipase variants towards unnatural substrates, such as carbohydrates. Mutant F344V showed a better reaction kinetics and/or regioselectivity then the wild type enzyme with several substrates while mutant F345V was inefficient in most cases. With the aim of correlating experimental data with the structural features of the enzyme and substrates, the interaction of substrates methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (**5a**) and 4,6-*O*-benzylidene-α-D-galactopyranoside (**6a**) with the wild type enzyme and the mutant F344V was investigated, using a molecular modelling approach.

Key Words: Biocatalysis; Lipases; Site-directed mutagenesis; Monosaccharides.

[†]This paper is dedicated to Professor Gérard Descotes on the occasion of his 70th birthday.

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INTRODUCTION

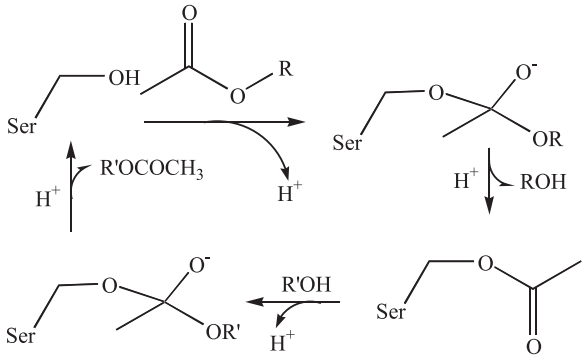
Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyse the hydrolysis of fats and oils.[1–4] In recent years lipases have received a great deal of attention as biocatalysts in numerous industrial processes.[5,6] They are the most widely used enzymes in synthetic organic chemistry, as catalyst of the chemo-, regio-, and/or stereoselective hydrolysis of carboxylic acid esters or of the reverse reaction in organic solvents.[6–8]

Recently, genetic engineering approaches have become powerful tools to improve enzyme performance,[9–12] in general. They have been largely applied in the search for more efficient enantioselective lipases.[13–16]

Lipases can acylate or deacylate hydroxyl groups present on carbohydrates (See Scheme 1). Their ability to discriminate among the available secondary hydroxyl groups in saccharides,[17] is particularly useful in organic synthesis. In general, lipases exhibit a preference for primary hydroxyl groups, and regioselective acylation at *O*-6 of glycopyranoses may be easily achieved.[18,19] In this context, we are interested in the search for lipase variants with modified regioselectivity properties towards monosaccharide derivatives. To this end, engineered forms of *Candida rugosa* lipase1 (CRL1) were tested on different monosaccharide derivatives.

RESULTS AND DISCUSSION

A synthetic gene coding for CRL1 was obtained by mutually priming long overlapping oligonucleotides. CUG codons were substituted with triplets coding for serine in order to enable its heterologous expression because of the nonuniversal use of CUG (serine instead of leucine) characteristic of *C. rugosa* genes.[20] The CRL1 active site is located inside a long hydrophobic substrate binding tunnel and may be either shielded or accessible to substrates according to the conformation of a mobile surface loop named the lid.[21] The substrate-binding site has been mapped by co-crystallization with substrate analogs.[22] Two bulky phenylalanine side chains F344 and F345 have been shown to protrude into the tunnel, reducing its size. Therefore, the wild-type



Scheme 1. Reaction mechanism of lipases; in the present study R = vinyl and R′ = monosaccharide.

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recombinant *lip1* gene was used as the template for PCR site-directed mutagenesis to replace phenylalanine residues 344 and 345 by valine.[23] Mutants were expressed in *Pichia pastoris* under the control of the methanol-inducible alcohol oxidase (AOX1) promoter. Fermentation in sorbitol-based minimal medium gave good yields of the recombinant proteins (0.6 mg/mL for the mutant F344V and 0.2 mg/mL for the mutant F345V) in the culture supernatants, from which they could be easily recovered, concentrated and lyophilised. The use of the minimal medium reduced the secretion of proteins other than the recombinant product, thus allowing the direct use of lyophilized culture supernatant in the reactions of interest. The variant F344V was secreted to levels comparable to the wild type recombinant protein and displayed similar activity in standard hydrolysis reactions (not shown), whereas both the secretion and the activity of mutant F345V were reduced. The effect of introducing substitutions in recombinant proteins is still largely unpredictable, since it can result in misfolding or impaired transport through the secretory pathway of the host's organism.[24] In fact, the sensitivity of the *P. pastoris* expression system to the amino acid sequence of heterologous proteins, and in particular rCRL, has been often noticed in our laboratory. Indeed, the replacement of residues that do not play any obvious role in the folding or activity of this protein can strongly decrease or even abolish its production.[25]

In order to study the different factors that can influence the regioselectivity of the acylation the lipase mutants F344V, F345V and the wild-type lipase (CRL), were tested for their ability to acylate a set of monosaccharides derivatised at their anomeric and primary hydroxyl groups.

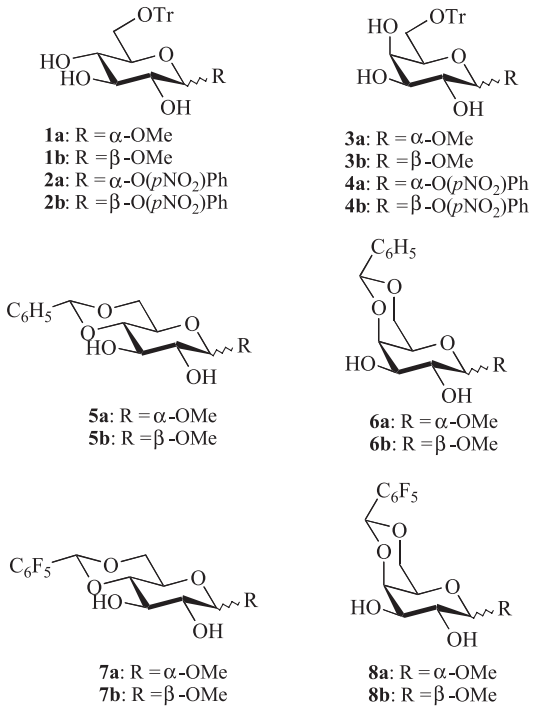

Figure 1. Substrates acylated by the WT-CRL, F344V and F345V mutants.

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Several parameters were investigated such as 1) the nature and orientation of the anomeric substituent, 2) the bulkiness of the *O*-6 substituent, 3) and the stereochemistry at C-4 as well as the blocking of the corresponding hydroxyl group (Figure 1). All reactions were carried out in vinyl acetate, which by acting both as the solvent and the acyl donor rendered the reaction completely irreversible.[26]

Initially, acylation of methyl 6-*O*-tritylglycosides **1**[27] and **3**[27] was studied using CRL and the mutated enzymes F344V and F345V (Table 1). The regioselectivity of the enzymatic acylation was determined from ^{1}H NMR and COSY experiments (Table 2). Analysis of the reaction kinetics indicated that α-anomers are better substrates of both CRL and the F344V mutant than the β-ones; in both cases, acylation took place at

Table 1. Enzymatic acetylation of partially protected monosaccharide derivatives using CRL, and mutants F344V and F345V.

Compd.	Enzyme	React. time (% conv.)[a]	% acylation in position 2
1a	CRL	36 h	> 98
	F344V	14 h	> 98
	F345V	1 month (10%)	> 98
1b	CRL	10 days	90
	F344V	10 days	> 98
	F345V	1 month (10%)	> 98
3a	CRL	14 h	> 98
	F344V	14 h	> 98
	F345V	1 month (10%)	n.d.
3b	CRL	10 days	92
	F344V	10 days	95
	F345V	1 month (10%)	n.d.
5a	CRL	3 days	> 98
	F344V	3 days	> 98
	F345V	40 days (10%)	n.d.
5b	CRL	10 days	70
	F344V	10 days	> 98
	F345V	40 days (10%)	n.d.
6a	CRL	10 days	90
	F344V	5 days	> 98
	F345V	40 days (10%)	n.d.
6b	CRL	n.r.	–
	F344V	10 days	> 98
	F345V	40 days (10%)	n.d.
7a	CRL	5 days	> 98
	F344V	n.r.	–
	F345V	n.r.	–
7b	CRL	5 days	> 98
	F344V	n.r.	–
	F345V	n.r.	–

n.r. = no reaction; n.d. = not determined.
[a]The percentage of conversion is 100% when not otherwise stated.

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Table 2. ^{1}H chemical shifts (δ) for monoacetylated derivatives obtained in the enzymatic reaction; *J* values (Hz) and signal multiplicity are reported within brackets; aromatic signals are omitted.

Compd.	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	CH_3CO	OH	OCH_3	C*H*-Ar
1a-2-*O*-Ac[26]	4.91 (d, 3.6)	4.72 (dd, 10.0; 3.6)	3.92 (dd, 10.0; 8.8)	3.58 (t, 8.8)	3.68–3.64 (m)	3.43–3.38 (m)	3.58–3.51 (m)	2.08 (s)	2.70 (bs)	3.38 (s)	–
1b-2-*O*-Ac[26]	4.27 (d, 7.8)	4.73 (dd, 9.3; 7.8)	3.57–3.32 (m)	3.57–3.32 (m)	3.57–3.32 (m)	3.57–3.32 (m)	3.57–3.32 (m)	2.17 (s)	2.82 (bs)	3.44 (s)	–
3a-2-*O*-Ac[26]	4.85 (d, 3.6)	4.98 (dd, 10.2; 3.6)	3.92 (dd, 10.2; 3.1)	4.05 (d, 3.1)	3.86–3.74 (m)	3.86–3.74 (m)	3.86–3.74 (m)	2.12 (s)	2.62 (bs)	3.31 (s)	–
3b-2-*O*-Ac[26]	4.21 (d, 8.0)	4.88 (dd, 9.7; 8.0)	3.56–3.36 (m)	3.94 (d, 3.3)	3.56–3.36 (m)	3.56–3.36 (m)	3.56–3.36 (m)	2.08 (s)	1.60 (bs)	3.44 (s)	–
5a-2-*O*-Ac[25]	4.94 (d, 3.7)	4.80 (dd, 9.7; 3.7)	4.17 (t, 9.7)	3.55 (t, 9.7)	3.84 (dt, 9.7; 4.6)	4.28 (dd, 9.7; 4.6)	3.75 (t, 9.7)	2.17 (s)	1.60 (bs)	3.40 (bs)	5.57 (s)
5b-2-*O*-Ac[25]	4.38 (d, 7.5)	4.85 (dd, 9.3; 7.5)	3.79–3.68 (m)	3.58–3.35 (m)	3.58–3.35 (m)	4.38 (dd, 9.7; 5.4)	3.79–3.68 (m)	2.18 (s)	3.12 (bs)	3.46 (s)	5.48 (s)
6a-2-*O*-Ac[25]	4.97 (d, 3.5)	5.15 (dd, 3.5; 10.3)	4.12–4.06 (m)	3.72 (bs)	4.30–4.27 (m)	4.12–4.06 (m)	4.30–4.27 (m)	2.16 (s)	3.82 (bs)	3.40 (s)	5.58 (s)
6b-2-*O*-Ac[25]	4.25 (d, 8.1)	5.12 (dd, 9.6; 8.1)	3.68 (dd, 9.6; 2.5)	4.19 (d, 2.5)	3.50 (bs)	4.38 (bd, 10.6)	4.10 (bd, 10.6)	2.13 (s)	3.42 (bs)	3.53 (s)	5.56 (s)
7a-2-*O*-Ac	4.94 (d, 3.7)	4.74 (dd, 9.6; 3.7)	4.14 (t, 9.6)	3.53 (d, 9.6)	3.86 (dt, 9.6; 4.7)	4.27 (dd, 9.6; 4.7)	3.71 (t, 9.6)	2.18 (s)	2.48 (bs)	3.41 (s)	5.95 (s)
7b-2-*O*-Ac	4.44 (d, 7.9)	4.85 (dd, 9.7; 7.9)	3.88 (t, 9.7)	3.58–3.51 (m)	3.58–3.51 (m)	4.34 (dd, 9.7; 4.6)	3.75 (t, 9.7)	2.12 (s)	2.72 (bs)	3.50 (s)	5.98 (s)

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position 2 (Table 1); it's worth noting that the regioselectivity of β-glucosides acylation was slightly improved when using mutant F344V, instead of the wild-type enzyme, but reaction rate was conserved. No improvement of the regioselectivity was observed in the case of mutated lipase F345V. Besides, the reaction time was extremely long, hence useless for synthetic purposes.

Acylation of the *p*-nitrophenyl glycosides **2** and **4** was studied in order to get better insight into the influence of the bulkiness of the anomeric aglycon on the lipase activity. Data (Table 1) showed that the concomitant presence of both the 6-*O*-trityl group and the anomeric *p*-nitrophenyl group prevented acylation, presumably interfering with the binding of the substrate. When the known 4,6-*O*-benzylidene glycosides **5**[28] and **6**[28] were tested as substrates, the α-anomers were strongly preferred by the wild-type lipase and its mutant F344V. Regarding mutant F345V, reaction times were, again, too long in order to be of synthetic utility (5–10% of conversion after 40 days). Comparing the CRL and the mutant F344V, it was observed that acylation with the wild-type enzyme was totally regioselective for position 2 only when methyl 4,6-*O*-benzylidene-α-D-glucopyranoside was the substrate. Again, the reaction rate was favored in the case of α-glycosides (3 and 10 days reaction time for **5a** and **6a**, respectively, versus 10 days for **5b** and no reaction for **6b**). On the contrary, although maintaining a strong preference for α-glycosides (3 and 5 days reaction time for **5a** and **6a**, respectively, versus 10 days for **5b** and **6b**), F344V showed improved reaction rate and regioselectivity towards all benzylidene glycosides **5**–**6** (Table 1). In other words, F344V is less substrate-specific compared to the wild-type enzyme (substrate **6b** could be acylated with the mutated lipase), but, at the same time, catalyses the acylation reaction with higher regioselectivity.

Finally, in order to elucidate if the minor substrate specificity, thus observed, could be ascribed to the enlargement of the catalytic pocket, or if polarity effects had to be taken into account, compounds **7** and **8** were synthesized. In this case, only the glucose derivatives were acylated by the wild-type enzyme, with 1) total regioselection for position 2, and 2) no apparent discrimination between anomers. The discriminating factor seemed to be the configuration at the 4-OH. In that case, the mutants failed to acylate the potential substrates.

With the aim of correlating some experimental data with the structural features of enzyme and substrates, we have investigated the interaction of substrates **5a** and **6a** with the wild type enzyme and the mutant F344V, using a molecular modelling approach.

To find acceptable starting configurations of the sugar-protein adducts, different binding modes of the substrates have been investigated by: 1) manually docking the substrates in the hydrophobic funnel leading to the active site, 2) monitoring the intermolecular energy as defined by Van der Waals and Coulomb terms, and 3) taking into account that the anionic oxygen of the tetrahedral intermediate has to be directed toward the oxyanion hole formed by the backbone NH atoms of Gly124 and Ala 210. In fact, following the computational approach proposed by Manetti et al.,[23] we have assumed that the different catalytic behaviours of the wild type enzyme and the mutant F344V depend mainly on the stability and structural characteristics of the tetrahedral intermediate formed along the reaction path. Remarkably, docking modes derived clustering results obtained for each protein-adduct are extremely similar and the only small differences are associated to rotation of the phenyl ring of the substrate (data not shown). The increase of the maximum temperature in the simulated annealing pro-

cedure to 1000 K did not affect results, indicating that the observed limited conformational freedom of the substrate does not depend on the adopted methodology but reflects the tight steric demand of the active site. However, significant differences are observed when results for different protein-substrate adducts are compared. The most stable structures of the enzyme-substrate adducts, as obtained by the simulated annealing approach described in Methods, are shown in Figure 2.

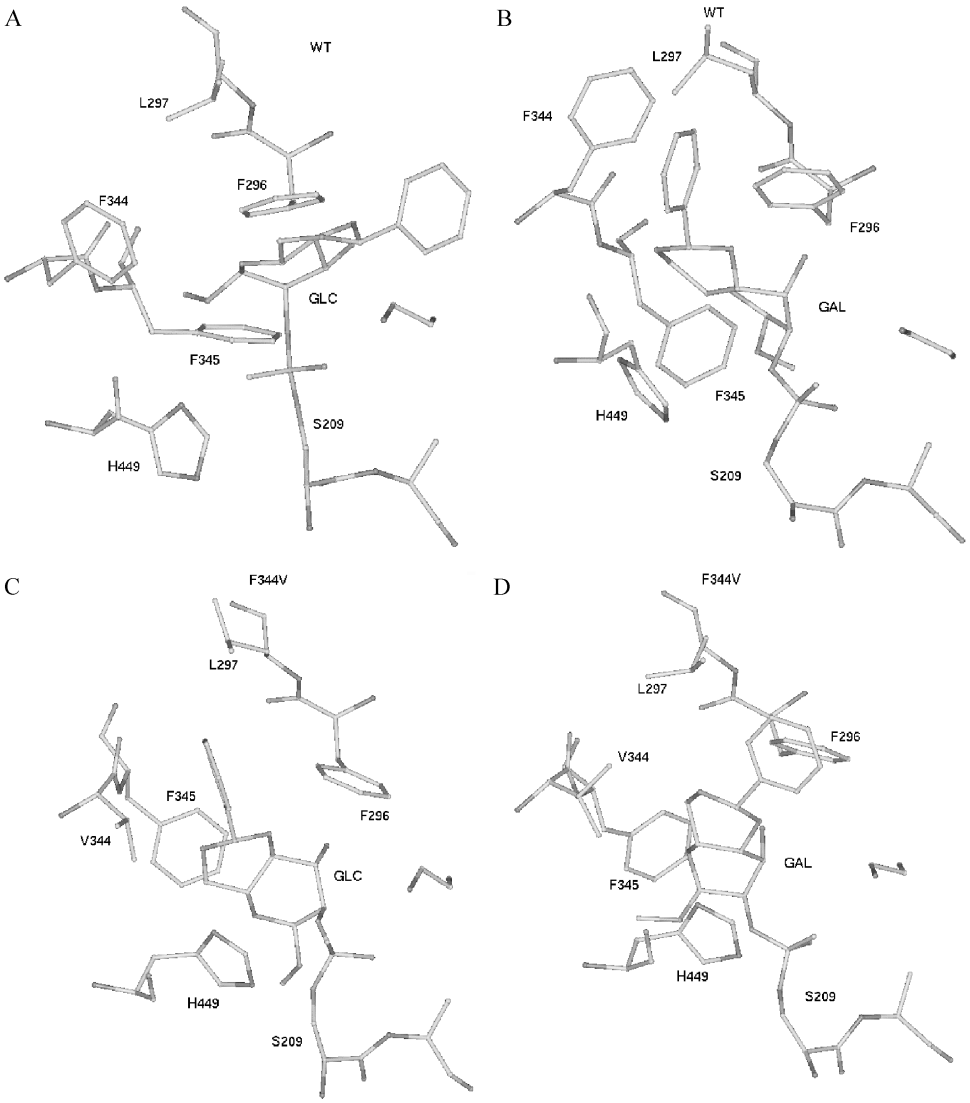

Figure 2. The most stable structures of the enzyme-substrate adducts, as obtained by the simulated annealing approach described in Methods. (A) CRL-**5a**; (B) CRL-**6a**; (C) F344V-**5a**; (D) F344V-**6a**. The figure was prepared using the software package InsightII [Biosym/MSI 9685, Scranton Road, San Diego, CA, USA]. *(Go to www.dekker.com to view this figure in color.)*

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Considering the structure of the active site, the substitution of phenylalanine 344 with valine results, as expected, in a larger pocket, decreasing the steric demands imposed by the enzyme.

Investigations of Wild Type Lipase-Substrate 5a Adduct

Surprisingly, in the optimized structure of this adduct (Figure 2A) the negatively charged oxygen atom of the tetrahedral intermediate weakly interacts with the oxyanion cavity of the enzyme defined by the backbone NH groups of residues 123, 124 and 210. The sugar ring and the phenyl group are largely solvent exposed and affect only slightly the orientation of Phe344 and Phe345. Residues Phe296 and Phe345 stabilize the complex by hydrophobic interactions with the sugar ring.

Investigations of Wild Type Lipase-Substrate 6a Adduct

In this adduct, the oxyanion cavity strongly interacts with the substrate, with the NH groups of residues 123, 124 and 210 at a distance ranging from 2.9 to 3.5 Å from the charged oxygen. The sugar ring induces a large conformational rearrangement of Phe344 and Phe345, to accommodate the substrate. Moreover, Phe296, Leu297, Phe344 and Phe345 form a hydrophobic cluster that interacts with galactose through stacking interactions (Figure 2B). This is the only case where the enzyme structure is strongly affected by the presence of the substrates.

Investigations of Mutant F344V-Substrate 5a Adduct

In this adduct, the negatively charged oxygen of the tetrahedral intermediate strongly interacts with the oxyanion cavity. Moreover, the substitution of Val for Phe344 enlarges the enzyme binding pocket and, as a consequence, the substrate can interact with the hydrophobic pocket formed by Phe296, Leu297, Val344 and Phe345 (Figure 2C).

Investigations of Mutant F344V-Substrate 6a Adduct

In this adduct, only weak hydrogen bonds between the substrate and the oxyanion cavity can be formed. The local structure of the enzyme is similar to that observed for the F344V—5a adduct. However, the substrate cannot fit properly in the hydrophobic pocket formed by Phe296, Leu297, Val344 and Phe345 (Figure 2D), interacting only with residues 296 and 345.

Remarkably, the structural analysis of these adducts reveals that the interaction between the substrate and the protein involves mainly hydrophobic and van der Waals interactions. This suggests that the very different reactivity of the fluoro-derivatives **7** and **8**, with respect to **5** and **6**, may depend on electronic interactions, such as hydrogen bonds, taking place during the initial recognition of the substrate by the enzyme.

Several considerations can be drawn from these studies: both the commercial, and mutated F344V enzymes generally showed a sharp preference for α-glycosides; this substrate specificity accounts for the different reaction kinetic, rendering the acylation faster for α-substrates compared to their β-counterparts. The larger binding pocket of

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the mutated enzyme F344V accounts for the minor substrate specificity, as confirmed also by molecular modelling. In addition, different interactions between the relevant residues in the oxyanion hole with the substrates play a fundamental role in the determination of the regioselection; this effect is clearly evidenced for the enzymatic reaction of **6a** with wild-type enzyme and F344V mutant. No discrimination of the anomers could be observed for compounds **7a** and **7b**, since the enzymes accepted both the α- and β-pentafluorobenzylidene glucosides; on the contrary neither anomer of galactosides (substrates **8a** and **8b**) could be acylated. In this case, the hydrophobic and van der Waals interactions are probably responsible for the discrimination between glucosides and galactosides.

Concerning mutant F345V, all enzymatic reactions were extremely slow, making this mutant unsuitable for biocatalyzed acylation.

Finally, it is worth noting that the glycoside anomeric configuration didn't influence the regiochemistry of the acylation reaction, contrary to similar experiments reported with lipase from *Pseudomonas cepacia*.[27]

EXPERIMENTAL

General methods. Solvents were dried over molecular sieves, for at least 24 h prior to use. Anhydrous reactions were performed under an Ar atmosphere. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Merck) with detection with UV light when possible, or charring with a solution containing conc. H_2SO_4/EtOH/H_2O in a ratio of 5/45/45. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). Optical rotations were measured at room temperature with a Perkin–Elmer 241 polarimeter. NMR spectra were recorded at 400 MHz on a Varian Mercury instrument using $CDCl_3$ as solvent unless otherwise stated. Chemical shifts are reported in ppm downfield from TMS as an internal standard; aromatic signals are omitted. CRL was purchased from SIGMA.

Synthesis of substrates. Substrates **1**, **3**, **5**, **6** were synthesised according to standard procedures,[27,28] starting from the commercially available glycosides.

General procedure for the synthesis of compounds 2, 4. *p*-Nitrophenyl glycosides were dissolved in dry pyridine, then trityl chloride was added. The reaction mixtures were allowed to stir for 48 h, then the reaction mixture was concentrated to dryness under reduced pressure. The crude material was purified by flash chromatography.

General procedure for the synthesis of compounds 7–8. Methyl glycosides (1 g, 5.15 mmol) were dissolved in dry acetonitrile (20 mL), then pentafluorobenzaldehyde (1.51 g, 7.73 mmol) and a catalytic amount of camphorsulfphonic acid (CSA, 24 mg, 0.10 mmol) were added. The reaction mixtures were allowed to stir overnight, then the reaction mixture was diluted with AcOEt, and washed with a sat. aq. $NaHCO_3$ solution. The organic layer was dried over Na_2SO_4, filtered and concentrated to dryness under reduced pressure. The crude was purified by flash chromatography.

Enzymatic acetylation reaction. The enzymatic reaction was performed by dissolving the substrate (10 mg) in vinyl acetate (0.5 mL), and incubating with the

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different lipases (10 mg) with shaking at rt. The conversion was monitored by TLC. The enzyme was removed by filtration, and the solvent evaporated to dryness under reduced pressure. The regioselectivity was then determined by ^{1}H NMR.

***p*-Nitrophenyl 6-*O*-trityl-α-D-glucopyranoside (2a).** *p*-Nitrophenyl α-D-glucopyranoside (50 mg, 0.166 mmol) was reacted with trityl chloride (93 mg, 0.33 mmol), in dry pyridine (1 mL) following the general procedure. After purification by flash chromatography (eluent: pure AcOEt) afforded 76 mg of **2a** (84%) as a colourless oil. $[\alpha]_D$ + 31.9 (*c* 1.5, $CHCl_3$); ^{1}H NMR: δ 5.60 (d, 1 H, *J* = 3.4 Hz, H-1), 3.95 (t, 1 H, *J* = 9.0 Hz, H-3), 3.74 (dd, 1 H, *J* = 9.0, 3.4 Hz, H-2), 3.72–3.68 (m 1 H, H-5), 3.54 (t, 1 H, *J* = 9.0 Hz, H-4), 3.37 (dd, 1 H, *J* = 10.4, 3.2 Hz, H-6a), 3.27 (dd, 1 H, *J* = 10.4, 5.7 Hz, H-6b), 2.30 (bs, 2 H, OH), 1.70 (bs, 1 H, OH).

Anal. Calcd for $C_{31}H_{29}NO_8$: C, 64.50; H, 5.38; N, 2.58. Found: C, 64.56; H, 5.41; N, 2.55.

***p*-Nitrophenyl 6-*O*-trityl-β-D-glucopyranoside (2b).** *p*-Nitrophenyl β-D-glucopyranoside (100 mg, 0.33 mmol) was reacted with trityl chloride (186 mg, 0.66 mmol), in dry pyridine (1.5 mL) following the general procedure. After purification by flash chromatography (eluent: petroleum ether:AcOEt 2:8) afforded 140 mg of **2b** (78%) as a colourless oil. $[\alpha]_D$ − 22.2 (*c* 2, $CHCl_3$); ^{1}H NMR: δ 4.86 (d, 1 H, *J* = 9.5 Hz, H-1), 3.61 (t, 1 H, *J* = 9.5 Hz, H-2), 3.57 (t, 1 H, *J* = 9.5 Hz, H-3), 3.50 (t, 1 H, *J* = 9.5 Hz, H-4), 3.47–3.44 (m, 1 H, H-5), 3.39 (t, 1 H, *J* = 9.5 Hz, H-6a), 3.23 (dd, 1 H, *J* = 9.5, 6.2 Hz, H-6b), 2.40 (bs, 1 H, OH), 2.18 (bs, 2 H, OH).

Anal. Calcd for $C_{31}H_{29}NO_8$: C, 64.50; H, 5.38; N, 2.58. Found: C, 64.47; H, 5.37; N, 2.60.

***p*-Nitrophenyl 6-*O*-trityl-α-D-galactopyranoside (4a).** *p*-Nitrophenyl α-D-galactopyranoside (37 mg, 0.123 mmol) was reacted with trityl chloride (68 mg, 0.25 mmol), in dry pyridine (0.5 mL) following the general procedure. After purification by flash chromatography (eluent: petroleum ether:AcOEt 2:8) afforded 50 mg of **4a** (76%) as a colourless oil. $[\alpha]_D$ + 43.3 (*c* 0.75, $CHCl_3$); ^{1}H NMR: δ 5.65 (d, 1 H, *J* = 3.4 Hz, H-1), 4.00 (dd, 1 H, *J* = 9.4, 3.4 Hz, H-2), 3.94 (bs, 1 H, H-4), 3.88 (dd, 1 H, *J* = 9.4, 3.3 Hz, H-3), 3.74 (t, 1 H, *J* = 5.3 Hz, H-5), 3.39 (dd, 1 H, *J* = 10.2, 5.3 Hz, H-6a), 3.26 (dd, 1 H, *J* = 10.2, 5.3 Hz, H-6b), 2.75 (bs, 3 H, OH).

Anal. Calcd for $C_{31}H_{29}NO_8$: C, 64.50; H, 5.38; N, 2.58. Found: C, 64.53; H, 5.39; N, 2.57.

***p*-Nitrophenyl 6-*O*-trityl-β-D-galactopyranoside (4b).** *p*-Nitrophenyl β-D-galactopyranoside (100 mg, 0.33 mmol) was reacted with trityl chloride (186 mg, 0.66 mmol), in dry pyridine (1.5 mL) following the general procedure. After purification by flash chromatography (eluent: petroleum ether:AcOEt 2:8) afforded 150 mg of **4b** (84%) as a colourless oil. $[\alpha]_D$ + 33.2 (*c* 0.25, $CHCl_3$); ^{1}H NMR: δ 4.85 (d, 1 H, *J* = 7.6 Hz, H-1), 3.93–3.89 (m, 2 H, H-2, H-4), 3.59–3.55 (m, 2 H, H-3, H-5), 3.48 (dd, 1 H, *J* = 10.1, 6.6 Hz, H-6a), 3.31 (dd, 1 H, *J* = 10.1, 4.7 Hz, H-6b), 2.80 (bs, 1 H, OH), 1.50 (bs, 2 H, OH).

Anal. Calcd for $C_{31}H_{29}NO_8$: C, 64.50; H, 5.38; N, 2.58. Found: C, 64.52; H, 5.36; N, 2.57.

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Methyl 4,6-*O*-pentafluorobenzylidene-α-D-glucopyranoside (7a). The crude material was purified by flash chromatography, eluting with pure AcOEt. Compound **7a** (1.72 g) was obtained as a white solid (90%): mp 59–61°C, $[\alpha]_D$ + 74.6 (*c* 0.5, $CHCl_3$); ^{1}H NMR: δ 5.88 (s, 1 H, H-benzylidene), 4.78 (d, 1 H, *J* = 3.9 Hz, H-1), 4.27 (dd, 1 H, *J* = 9.8, 4.8 Hz, H-$6_{eq.}$), 3.92 (t, 1 H, *J* = 9.2 Hz, H-4), 3.84 (dt, 1 H, *J* = 9.8, 4.8 Hz, H-5), 3.70 (t, 1 H, *J* = 9.8 Hz, H-$6_{ax.}$), 3.50 (dd, 1 H, *J* = 9.8, 3.9 Hz, H-2), 3.48 (s, 3 H, OCH_3), 3.46 (t, 1 H, *J* = 9.8 Hz, H-3), 2.9 (bs, 1 H, OH), 2.4 (bs, 1 H, OH).

Anal. Calcd for $C_{14}F_5H_{13}O_6$: C, 45.17; F, 25.52; H, 3.52. Found, C, 45.21; F, 25.55; H, 3.51.

Methyl 4,6-*O*-pentafluorobenzylidene-β-D-glucopyranoside (7b). Following the general procedure previously described, **7b** (1.67 g, 87%) was obtained as a white solid, after purification by flash chromatography (eluent: petroleum ether:AcOEt 2:8): mp 120–121°C, $[\alpha]_D$ − 45.8 (*c* 0.5, $CHCl_3$); ^{1}H NMR δ, 5.86 (s, 1 H, H-benzylidene), 4.32 (dd, 1 H, *J* = 9.9, 4.1 Hz, H-6_{eq}), 4.30 (d, 1 H, *J* = 7.5 Hz, H-1), 3.80 (t, 1 H, *J* = 9.9 Hz, H-3), 3.72 (t, 1 H, *J* = 9.9 Hz, H-6_{ax}), 3.55 (s, 3 H, OCH_3), 3.52–3.48 (m, 2 H, H-4 and H-5), 3.42 (dd, 1 H, *J* = 9.9, 7.5 Hz, H-2), 3.02 (bs, 2 H, OH).

Anal. Calcd for $C_{14}F_5H_{13}O_6$: C, 45.17; F, 25.52; H, 3.52. Found, C, 45.14; F, 25.54; H, 3.49.

Methyl 4,6-*O*-pentafluorobenzylidene-α-D-galactopyranoside (8a). Compound **8a** (1.57 g, 82%) was obtained as a white solid after purification by flash chromatography (petroleum ether:AcOEt 2:8): mp 95–97°C, $[\alpha]_D$ + 102.0 (*c* 0.65, $CHCl_3$); ^{1}H NMR δ, 5.94 (s, 1 H, H-benzylidene), 4.98 (d, 1 H, *J* = 3.1 Hz, H-1), 4.30 (dd, 1 H, *J* = 12.5, 1.5 Hz, H-6a), 4.25 (bd, 1 H, *J* = 2.2 Hz, H-4), 4.05 (dd, 1 H, *J* = 12.5, 1.5 Hz, H-6b), 3.94–3.92 (m, 2 H, H-2 and H-3), 3.72 (bd, 1 H, *J* = 7.5, 1.5 Hz, H-5), 3.47 (s, 3 H, OCH_3), 2.50 (bs, 1 H, OH), 2.22 (bs, 1 H, OH).

Anal. Calcd for $C_{14}F_5H_{13}O_6$: C, 45.17; F, 25.52; H, 3.52. Found, C, 45.19; F, 25.50; H, 3.54.

Methyl 4,6-*O*-pentafluorobenzylidene-β-D galactopyranoside (8b). Compound **8b** (1.74 g, 91%) was obtained as a white solid after purification by flash chromatography (petroleum ether:AcOEt 2:8): mp 108–110°C, $[\alpha]_D$ − 20.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR δ, 5.96 (s, 1 H, H-benzylidene), 4.34(bd, 1 H, *J* = 12.5 Hz, H-6a), 4.22 (d, 1 H, *J* = 8.2 Hz, H-1), 4.19 (bd, 1 H, *J* = 2.6 Hz, H-4), 4.05 (bd, 1 H, *J* = 12.5 Hz, h-6b), 3.77 (t, 1 H, *J* = 8.2 Hz, H-2), 3.71 (dd, 1 H, *J* = 8.2, 2.6 Hz, H-3), 3.59 (s, 3 H, OCH_3), 3.51 (bs, 1 H, H-5), 2.81 (bs, 1 H, OH), 1.98 (bs, 1 H, OH).

Anal. Calcd for $C_{14}F_5H_{13}O_6$: C, 45.17; F, 25.52; H, 3.52. Found, C, 45.15; F, 25.48; H, 3.53.

Enzyme production. Lipase variants were produced through fermentation of *P. pastoris* strain X-33 transformed with the expression plasmid pPICZαB (Invitrogen Corporation, San Diego, CA) carrying the mutated lipase genes.[24] Cells were grown in shaking flasks at 30°C in minimal medium containing 2% sorbitol, 1.34 g/L yeast nitrogen base in phosphate buffer, pH 6.0. Cells were grown until a density of 10^8 cells/mL was reached, and then induced by daily addition of 1% v/v methanol. After 5

days, induction cells were harvested by centrifugation at 11.000 rpm for 45 min. The supernatant was filtered through 0.45 μm cut-off filters (Millipore) and concentrated by tangential flow filtration against 4–5 volumes of 5 mM Tris-HCl pH 7.5 with a Minitan system (Millipore Co., Bedford, MA, USA) using filter plates at 60 kDa exclusion. Mutant enzymes were identified by electrophoresis on 10% SDS-polyacrylamide gel,[29] and quantified by comparison with standard BSA at known concentrations. Determination of total proteins was according to Bradford.[30] Lipase was quantified by densitometry analysis.

Molecular modelling. Calculations and graphical manipulations were carried out on a Silicon Graphics Indigo workstation using the software package InsightII [Biosym/MSI 9685 Scranton Road, San Diego, CA, USA]. Molecular mechanics and dynamics calculations were performed using the Consistent Valence Force Field.[31]

The structure of the enzyme used in this study (CRL) was derived from the Brookhaven Protein Data Bank. The potential energy surface has been scanned, with the aim of locating low energy structures, using molecular dynamics and simulated annealing approaches. Due to the extremely large number of atoms, some degrees of freedom have been frozen during the simulations. In particular, final structures have been obtained according to the following procedure. The structure of the starting enzyme-substrate adduct was obtained by freezing all atoms but the substrate and the amino acids directly interacting with it (residues 296, 297, 344, 345); then the optimization was carried out by unfreezing all residue within 8 Å from the substrate and finally performing 25 cycles of simulated annealing. Each simulated annealing cycle is made by 25 picoseconds (ps) of molecular dynamics where the temperature is raised from 50 to 500 K, 100 ps of molecular dynamics at 500 K and 150 ps of molecular dynamics where the temperature is decreased to 50 K. The time step was set to 0.001 ps. The structures obtained from the simulated annealing procedure have been optimized and clustered according to structure similarity (root mean square displacement).

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